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Phylogenetic analysis of 3' region of Helicobacter pylori cagA gene of Lombok isolates and the association with gastric pathology

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Abstract: *Lombok island is a transitional region beetwen Asian and Australian faunal biodiversity. There has been no previous report on the genotype of Helicobacter pylori in the population. The cagA gene is one of the important virulence factors of H. pylori. The diversity of cagA 3′ regions reflect the phylogenetic relationships among the different H. pylori isolates and their association with clinical outcomes and significant geographical differences among isolates have been reported. The purpose of this study was to analyse the variability of 3'region of cagA H. pylori Lombok isolates and determine the genotype based on EPIYA(Glu-Pro-Ile-Tyr-Ala) motif of CagA and its relationship with pathological of the stomach. Total of 36 isolates of H. pylori Lombok origins were studied. Gastric antral biopsies collected from 36 Lomboknese patients (chronic gastritis = 13, gastric/duodenal ulcer =12, and gastric cancer =10) were used to amplify the 3' regions of cagA containing EPIYA motifs by PCR then followed by DNA sequencing. We have found that 34/36 of the H. pylori Lombok isolates were clustered alone while 1/36 was clustered together with other East Asian strains. The findings of the study suggested that Lombok H. pylori strains share a common ancestry with East Asians but with their own unique sequences. No association of specific sequences with the outcome of disease was revealed through additional phylogenetic analysis based on amino acid sequences.*

Keywords: *Helicobacter pylori, Phylogenetic analysis, cagA, EPIYA*

I. Introduction

Lombok island is located east of two most populous islands in Indonesia, Java and Bali, and that as a transition zone between the Asian and the Australian faunal biodiversity. There has been no previous report on the genotype of *Helicobacter pylori* in the population. *Helicobacter pylori* possess several virulence factors that play important role in the development of gastric diseases such as chronic gastric inflammation, ulcerative peptic diseases, gastric cancer and MALT-lymphoma [1]. There are many virulence factors for *H. pylori* colonization and infection but CagA is one of the most studied virulence of *H. pylori*. This toxin is encoded by cagA gene located on cagPAI, and protein CagA 120–145 kDa in size [2]. *H. pylori* strains which carry the cagPAI are known to be more virulent than those that do not. After CagA injected into the host cell by the type IV secretion system (T4SS), CagA exerts its effects directly on gastric mucosal cells [3,4,5].

Type 1 cagA-positive *H. pylori* strains have varies prevalences among different populations. In many East Asian countries (with high prevelences of gastric cancer), isolated strains were dominated by cagA-positive but in Western countries (low prevalences gastric cancer) this frequency is much lower [6]. The cagA genes structure reveals a 5′ highly stable region and a 3′ variable region. The variation size of the CagA protein has been correlated with the varying number of copy and sequences type located in the 3′ variable region of the gene that encodes the EPIYA motifs [7]. Based on the types of these motifs, CagA and *H. pylori* were divided into (i) Western and (ii) East Asian type. The percentage of homology between the CagA repeat sequences of Western and East Asian strains less than 53%. This indicates the existence of gene variability that might affect the toxin strength [8, 9].

The association between *H. pylori* and human has been predicted since the existence of human ancestors in Africa about sixty thousand years ago [10]. and *H. pylori* genetic appears to be a reflection of thousands years evolution. Variability of cagA gene among strains from different geographic regions has been analyzed [11, 12]. The tyrosine phosphorylation motifs (TPM) contain the Glu-Pro-Ile-Tyr-Ala (EPIYA) sequence were used to determine sequence diversity. While the phylogenetic analysis performed on a large part of the *cagA* gene [13, 14]. Cortes *et al* [15] did phylogenetic analyses based on the deduced amino acid sequence of CagA and its indicated that the Philippine strains were classified into the two major groups of CagA protein: the East Asian and the Western group. No East Asian (EPIYA-ABD type) was found in the *H. pylori* strains isolated from Brazilian population [16] and India [17]. In this research, we analyzed phylogenetic of the cagA positive strains in Lombok. The EPIYA pattern of the cagA and strain type of *H. pylori* was established

and evaluated whether its associated with gastroduodenal diseases. We hope the data obtained helped us to revealing the interrelationship between these virulence factors with pathogenesis of gastric ulceration and cancer in unique remote area, Lombok island.

II. Methods

2.1. *H. pylori* Strains

H. pylori Lombok isolates provided from Microbiology Laboratory, Biomedical Research Unit, West Nusatenggara General Hospital, which have been isolated from gastric antral biopsies collected from 36 Sasak Lomboknese patients (Gastritis = 13, Gastric/Duodenal Ulcer = 12, Gastric Cancer = 11) and stored at -80 ^{o} C. Medical records of patients provided from Endoscopy Unit, West Nusatenggara General Hospital at Mataram, Lombok Indonesia.

2.2. Amplification of the 3' Region of the cagA Gene

Genomic DNA extraction was done using the DNAZol Kit (Invitrogen) according to the manufacturer′s instructions. Amplification of the cagA 3′ variable region was done by using the primers P1 (5′-GA TAACAGGCAAGCTTTTTGAGG-3′) and P2 (5′-CTGCAAAAGATTGTTTGGCAG-3′) [18]. The amplification steps were done under the following conditions: 94°C for 1min; 34 cycles of 94°C for1min 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.

2.3. Phylogenetic Analysis and Determination of the EPIYA Pattern

The analysis included cagA 3′ region nucleotide sequences of 44 isolates of which 36 were Lombok isolates and 8 from other countries retrieved from GeneBank. To determine the phylogenetic relationship between Lombok isolates and those from other countries, the nucleotide sequences of *cagA* 3′ region was multiple aligned and Maximum Likelihood phylogenetic tree constructed using the CLUSTALW program. To clarify the phylogenetic relationship among Lombok isolates from different gastric pathology, the nucleotide sequence translated into amino acid sequence. The translated 3′ region sequences of our Lombok 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 [20]. To predict structure of the C-terminal CagA protein we use SWISS-MODEL program [21].

III. Result

3.1. The Demographic and Geographic Characteristics

We collected 36 isolates of *H. pylori* were cultured from gastric biopsies of dyspepsia patients undergoing endoscopic examination at the West Nusatenggara Province General Hospital. Demographic characteristics of the subjects gastric disease patients showed in Table 1.

Tabel 1 Demographic characteristic

Characteristics	n (%)
Sex	
-Male	26 (72,2)
-Female	10 (27,8)
Age :	
- < 30 year	4 (11,1)
- 30-50 year	26(72,2)
- >50 year	6 (16,7)
Ethnicity :	
- Sasak (Lomboknese)	33 (91,7)
- Balinese	2 (5,6)
- other	1(2,7)
Endoscopy Diagnosis :	
- Chronic Gastritis	13 (36,1%)
- Gastric or Duodenal Ulcer (DU)	12 (33,3%)
- Gastric Cancer (GC)	11 (30,6%)

The average age of the patients was 42.19 year-old. The representation per age group was 4 (11,1%) patients under 30 years; 26 patients (72,2%) between 30 and 50; and 6 patients (16,7%) were above 50. Most of the age of the patient is an adult so it is considered chronic *H. pylori* infection and gastric pathology associated with the condition. Lombok Island is located east of Java and Bali and biodiversity is somewhat different with the two most populous island in Indonesia.

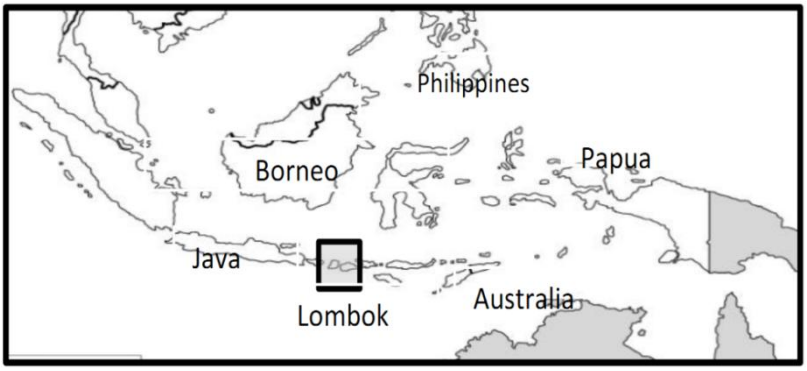

Figure 1 Lombok island map.

3.2. The prevalence of CagA gene

All of the 36 *H. pylori* isolates examined positive for *cagA* gene. Based on the result of the gel electrophoresis, a 660 bp single band observed similar in each sample. This is indicated low variation of 3' region cagA Lombok isolates.

3.3. *H. pylori* isolates comparison with neighboring countries

Phylogenetic analysis of 3' variable regions of *cagA* gene of *H. pylori* Lombok isolates compared with isolates from Japan, China, Korea, Philippines, Malaysia and Thailand show the separation Lombok isolates and form a separate cluster. While *H. pylori* reference isolate from the North Sulawesi, Indonesia comparative form a cluster with a Philippines strain. One Lombok isolate LOM-38 was gathered in sub-group with an Indian-Malay strain, which known as Western strain. As shown in Fig. 2, a big cluster Lombok isolates formed two sub-clusters, but it is not significant because the value of bootstrap only 64.

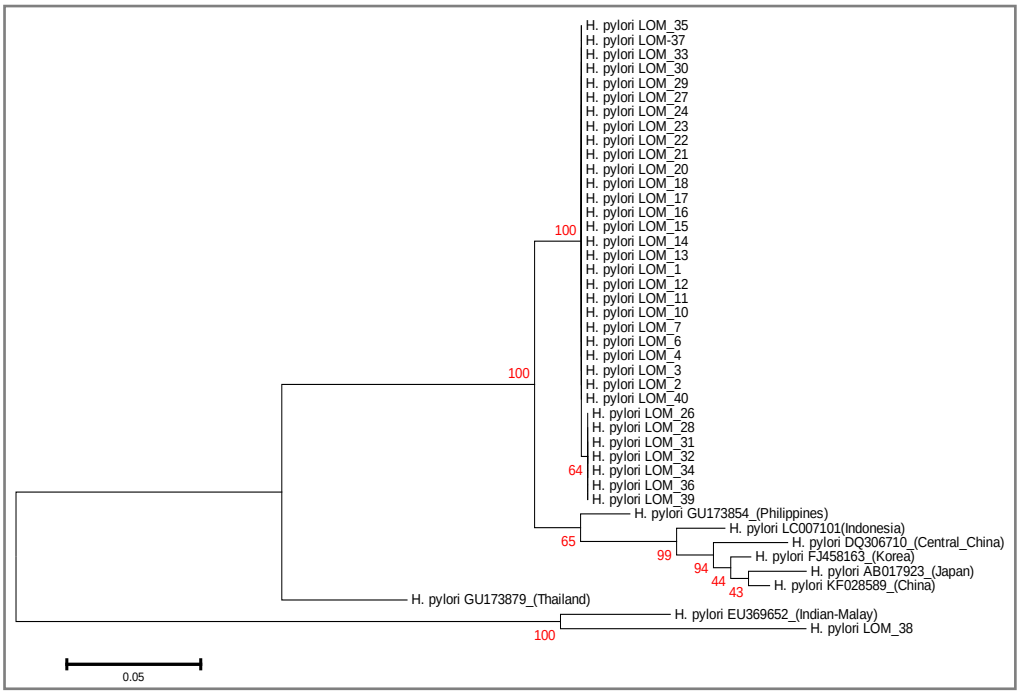

Figure 2. Phylogenetic tree based on the *cagA* 3' variable regions of *H. pylori* Lombok isolates compared with 8 reference strains from several East Asian countries with accesion number GU173854 (Philippines), DQ306710 (Central China), FJ458163 (Korea), AB017923 (Japan), KF028589 (China), GU173879 (Thailand), LC097101 (Indonesia / North Sulawesi) and EU369652 (Malaysia), retrieved from the GeneBank database.

4.4. CagA sequences Lombok Isolate and Pathology Stomach

CagA partial sequence at the C-terminal region is divergent and it contains EPIYA motifs. Results of phylogenetic tree construction of *H. pylori* using the amino acid sequences between Lombok isolates isolated from patients with different gastric diseases do not show any grouping according to the type of disease. Most of 34/36 isolates formed one cluster with the high similarity.

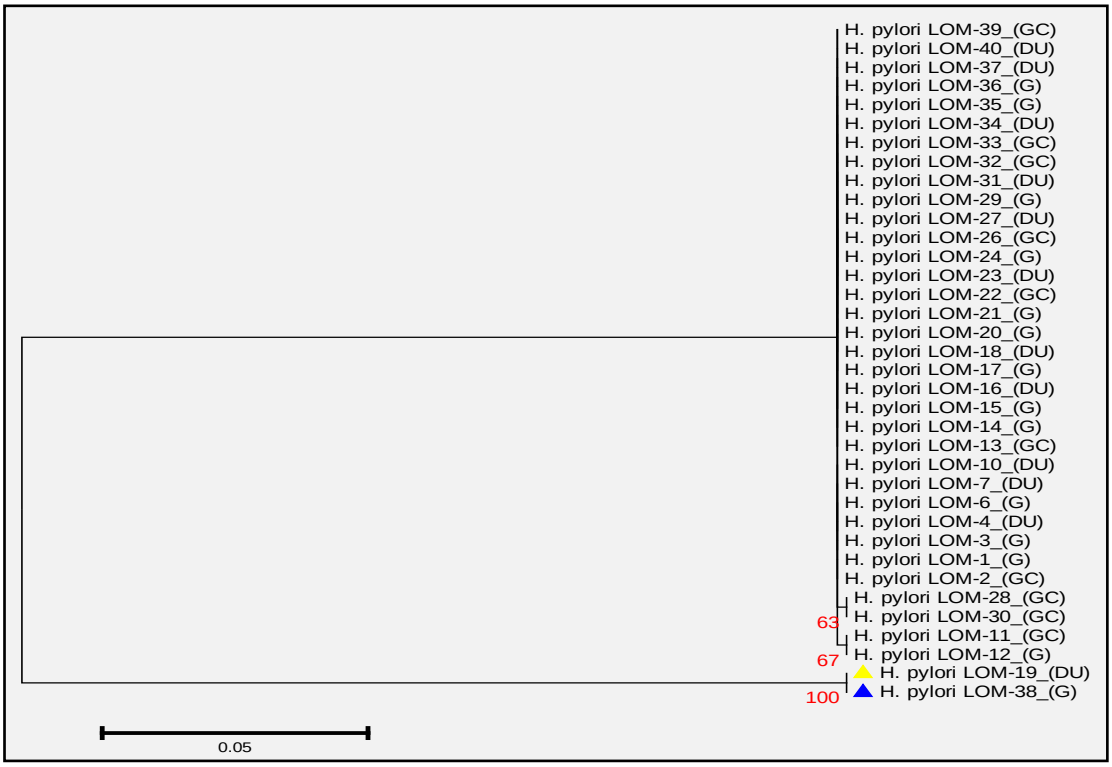

Figure 3. Phylogenetic tree of deduced amino acid sequences of cagA 3' variable regions among *H. pylori* Lombok isolates was built with Maximum Likehood. Red numerical indicate boostrap value. The letters in parentheses indicate the type of disease that is G gastritis, DU gastric or duodenal ulcer, and GC gastric cancer.

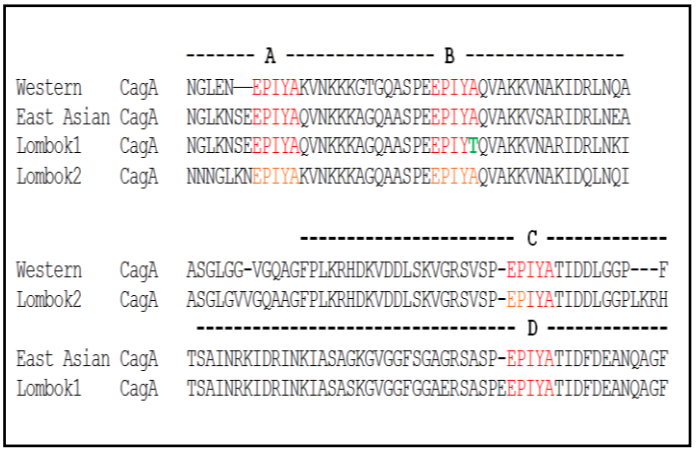

Figure 4 Alignments of the deduced amino acid sequence of the C-terminus of CagA between the Lombok1 and Lombok2 strain and the representative East Asian CagA and Western strains [3].

Low variation of the amino acid sequence C-terminus containing EPIYA motif of CagA *H. pylori* was found in Lombok isolates. Strain Lombok1 isolates (97,2%) were observed to have EPIYA-ABD motif and 2.8% is Lombok2 strain (2,8%) has EPIYA-ABC motif. There is a unique motifs EPIYT of segment-B on Lombok1 strain (Fig . 4. marked in green letters).

IV. Discussions

Many studies have shown infection with cagA–positive *H. pylori* strain to be closely associated with gastric cancer and peptic ulcers.[3,4]. Nonetheless, the majority of the *H. pylori* infected patients do not develop serious diseases. In the present study, all of the infecting strains harbored the cagA gene which is above the global prevalence of cagA–positive *H. pylori.* Around 90 to 95% of`*H. pylori* strains isolated in East Asian countries such as Japan, Korea and China carry cagA gene. In contrast, only 60% of *H. pylori* strains isolated in Western countries such as America and Africa carry cagA gene [3]

In this study, the majority (91.7%) patients are Sasak Lomboknese. It is expected that the *H. pylori* isolates studied are endemic and not the result of recently migration from outside of Lombok. In terms of the age of the patients, all of them are adults. As is well known that *H. pylori* infection generally starts from an early age [22], and the presence of *H. pylori* in the stomach of adult means is chronic and pathological conditions of the stomach observed.

Wallace's line put Lombok on the transition region between Asian and Australian faunal biodiversity. Sasak the original inhabitants of the island of Lombok is estimated to come from Java and Lombok island has been inhabited for thousands years [23].

The prevalence of *H. pylori* in Lombok is relatively higher [24] compared to Java (especially the middle) reported very low [25]. The high prevalence of *H. pylori* in Lombok is also accompanied by high positive CagA which means high risk for gastric ulcer and cancer.

EPIYA repetitive sequence in CagA has been used as the basis for determination of *H. pylori* genotype. Based on these genotypes, East Asian strains motif EPIYA-ABD considered more carcinogenic than a Western strain motif EPIYA-ABC. EPIYA type D binds stronger than type C. Complex SH2-SH2 CagA activates SHP2 which then affects cell proliferation through MAPK-Erk pathway [26]. In this study, 35 of 36 (97.2%) isolates were studied have East Asian strain, clustered with other East Asian strains with accesion number GU173854 (Philippines), LC097101 (Indonesia/North Sulawesi), DQ306710 (Central China), FJ458163 (Korea), AB017923 (Japan), KF028589 (China), GU173879 (Thailand) and EU369652 (Indian-Malaysia). Tree of phylogenetic as seen in Fig 2 showed that *H. pylori* Lombok isolates forming one sub-cluster which separate with a value of 100. While *H. pylori* reference isolate from northern part of Sulawesi (Minahasa) form a cluster with a Philippines isolate. As we know North Sulawesi located near Philippines teritory. One isolate Lombok is LOM-38 occupies a cluster of very different and include genotype Western commonly found in the Middle East, India and Europe. As shown in Fig 2, LOM-38 isolate to form a separate cluster along with isolate from Indian-Malay and both have the Western type.

V. Conclusion

Most of *H. pylori* Lombok isolates (97.2%) has a type of East Asian motif EPIYA-ABD. All of East Asian type *H. pylori* Lombok isolates have substitution at EPIYA into EPIYT. Need to do research to include *H. pylori* isolates from other geographic regions such as Java, Borneo, Timor and Papua. Research is needed to prove that EPIYA-ABD is more carcinogenic than EPIYA-ABC in which the D segment is considered more strongly affect the regulation of cell proliferation.

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