RESEARCH ARTICLE

Virulence Genes of *Helicobacter pylori* in Gastritis, Peptic Ulcer and Gastric Cancer in Laos

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Abstract

Background: *Helicobacter pylori* (*H. pylori*) infection is an established cause of peptic ulcers and gastric cancer. The aim of this study was to identify *H. pylori* genotypes and to examine their associations with geographical regions and gastritis, peptic ulcers and gastric cancer in Laos. Materials and Methods: A total of 329 Lao dyspeptic patients who underwent gastroscopy at Mahosot Hospital, Vientiane, Laos during December 2010 - March 2012 were enrolled. Two biopsy specimens (one each from the antrum and corpus) were obtained for CLO testing and only CLO test-positive gastric tissue were used to extract DNA. PCR and sequencing were identified for variants of the *cagA* and *vacA* genotypes. Results: Some 119 Laos patients (36.2%) were found to be infected with *H. pylori* including 83 with gastritis, 13 with gastric ulcers (GU), 20 with duodenal ulcers (DU) and 3 with gastric cancer. *cagA* was detected in 99.2%. East-Asian-type *cagA* (62%) and *vacA* s1c (64.7%) were predominant genotypes in Laos. *vacA* s1c-m1b was significantly higher in GU than gastritis (53.8% *vs.* 24.1%; P-value=0.04) whereas *vacA* s1a-m2 was significantly higher in DU than gastritis (40.0% *vs.* 16.9%; P-value=0.03). East-Asian-type *cagA* and *vacA* s1c were significantly higher in highland than lowland Lao (100% *vs.* 55.8%; P-value=0.001 and 88.2% *vs.* 61.5%, P-value=0.03 respectively). Conclusions: *H. pylori* is a common infection in Laos, as in other countries in Southeast Asia. The *cagA* gene was demonstrated in nearly all Laos patients, *cagA* and *vacA* genotypes being possible important factors in explaining *H. pylori* infection and disease outcomes in Laos.

Keywords: *Helicobacter pylori* - *cagA* gene - *vacA* gene - Laos

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Introduction

Helicobacter pylori (*H. pylori*) infection is now accepted as the major cause of chronic gastritis as well as severe diseases including peptic ulcers, gastric cancer and MALT lymphoma (Warren and Marshall., 1983; Dixon., 1991; Tytgat et al., 1993; Parsonnet et al., 1999; Wotherspoon et al., 1999; Sriamporn et al., 2002; Hamajima et al., 2004; Tokudome et al., 2006; Park et al., 2008; Pandey et al., 2011; Zheng et al., 2012). Although gastric cancer is one of the leading causes of cancer-related death, only a minority of individuals with *H. pylori* infection ever develops this particular cancer. The possible explanation might be related to differences in virulence factors of *H. pylori* strains in addition to host, environmental, and dietary factors. Human HLADQB1 genes might play important roles in *H. pylori* infection in Indonesian people (Zhao et al., 2012). Blood group O were likely to develop *H. pylori* infection related to peptic ulcers, peptic ulcer perforation in Pakistan (Valliani et al., 2013). Several *H. pylori* virulence factors, including *cagA* and *vacA*, have been reported to be associated with the clinical outcomes (Weel et al., 1996; Wotherspoon et al., 1999; Cettelly et al., 2002; Yamaoka., 2010). Recently, *H. pylori vacA* d1 genotype was reported to be associated with gastric cancer in Iraq (Hussein, 2014) and Iran (Basiri et al., 2014). Seroreactivity to the 89kDa (*vacA*) protein was also significantly higher in gastric cancer patients in Iran (Karami et al., 2014).

cagA gene has been proposed as a marker for a genomic pathogenicity island (*cag* PAI) with approximately 40 kbp whose presence is associated with more severe clinical outcomes (Blaser et al., 1995; Atherton., 2000). Several studies in Western countries have demonstrated that *cagA* gene was associated with peptic ulcer and gastric cancer (Well et all., 1996; Parsonnet et al., 1997; Rudi et al., 1998). In contrast, the majority of studies from East Asian countries demonstrated high prevalence of *cagA*-

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positive *H. pylori* without diseases association (Maeda et al., 1998; Kim et al., 2001). The 3' repeat region of the *cagA* can be used as a marker to separate East Asia from those in Western countries. Interestingly, studies from Asian countries reported that this East-Asian *cagA* might be a subgenotype of *cagA* gene which plays important roles in gastroduodenal disease development (Yamaoka et al., 1998; Yamaoka et al., 1999, Yamaoka et al., 2000; Yamaoka et al., 2002).

vacA is present in many of *H. pylori* strains. *vacA* genotypes were determined by the combination in the signal region (s1 and s2) and the middle (m1 and m2) region (Atherton et al., 1995). Furthermore, the *vacA* s1 subtype is divided into s1a, s1b and s1c (Atherton et al., 1995; Doorn et al., 1998) and the m1 subtype is divided into m1a, m1b and m1c (Mukhopadhyay et al., 2000). Even most strains of *H. pylori* contained *vacA* gene, active toxin was expressed in only approximately 50% (Cover et al., 1992). Among the s1 subtype, s1/m1 is more virulence for wider range of epithelial cells damage than s1/m2 (Letley et al., 2003), and the *vacA* s2/m2 strains are almost non-toxic (Atherton et al., 1995) and might be not related with diseases development (Atherton et al., 1997; Doorn et al., 1999; Atherton., 2006).

The Lao People Democratic Republic (Laos) is located in Southeast Asia; the North is border with China and Myanmar, the South is border with Cambodia, the East is border with Vietnam and the West is border with Thailand. Laos has population of 6.28 million people in 2011 (World Development Indicators 2012, World Bank database), classified into 3 major difference ethnic groups: Lao Lum (lowland Lao), Lao Thoeng (midland Lao) and Lao Sung (highland Lao). The gastric cancer was one of the cancer related death in Laos with ASR 6.9 and 7.1 in male and female respectively (Nguyen et al., 2011). A previous study reported that the prevalence of *H. pylori* infection in Laos was 68.4% with no differences according to age and sex. However, *H. pylori* infection was more common in patients with duodenal ulcers (80.0%) than those of normal endoscopic findings (60.6%). In addition, there was no prior study of *H. pylori* genotype related with clinical disease outcomes (Rasachak and Sihavong., 2000). Therefore, this study was aimed to investigate the prevalence of *cagA* and *vacA* genotypes of *H. pylori* and to examine the association between the theses genotypes and gastroduodenal diseases in Laos' dyspeptic patients.

Materials and Methods

Patients

Total of 329 Lao dypeptic patients who underwent gastroscopy at Mahosot Hospital, Vientiane, Laos during December 2010 - March 2012 were enrolled in this study. Inclusion criteria consisted of (1) age more than 15 years old, (2) had indication for upper gastrointestinal endoscopy, (3) no prior history of chronic severe medical illness such as chronic renal failure, congestive heart failure or HIV infection (4) no history of bleeding diathesis and (5) providing informed consent. Exclusions were (1) patient had received antibiotic or proton pump inhibitor within 1 month prior to the study, (2) had history of previous *H. pylori* eradication, and (3) had contraindication for gastric biopsy. Gastric biopsy specimens were taken each from antrum and corpus. Gastric ulcer (GU), duodenal ulcer (DU), and gastric cancer were identified by endoscopy, and gastric cancer was further confirmed by histopathology. Gastritis was defined as histological gastritis in the absence of peptic ulcer or gastric malignancy. The status of *H. pylori* infection was determined based on the result of rapid urease test (CLO test). Informed consent was obtained from all patients and the protocol was approved by the Ethics Committee of Lao National Ethics Committee for Health Research.

H. pylori genotyping

H. pylori DNA was extracted from gastric tissue in case the CLO-test yielded positive results. DNA was extracted using the QIAamp DNA Mini Kit (QIAGEN, Inc. Santa Clarita, CA, USA) according to the manufacturer's instructions. The *cagA* genotypes (East-Asian type or Western type) and *vacA* genotypes (s region: s1a, s1b and s1c and m regions: m1a, m1b, m1c and m2) were evaluated by polymerase chain reaction (PCR) as previously described (Atherton et al., 1995; Doorn et al., 1998; Yamaoka et al., 1998; Mukhopadhyay et al., 2000; Kersulyte et al., 2000; Ghose et al., 2002; Yamaoka et al., 2002). To confirm the results obtained by PCR and to determine the status of *vacA* m1c in case of *vacA* m1a, m1b and m2 were negative, PCR-based sequencing was also performed as previously described (Yamaoka et al., 2002). The PCR condition was 35 cycles consisting of 1 minute at 95°C, 1 minute at 52°C and 1 minute at 72°C. The final cycle included a 7-minutes extension step to ensure full extension of the PCR products. The amplified fragment was detected by a 1.5% agarose gel electrophoresis using an ultraviolet transilluminator. DNA direct sequencing was performed using AB 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

Data analyses

All results are expressed as frequency and percentage as appropriate. Fisher's exact test or the Chi-square test was used for analyzing of categorical data. A p-value<0.05 was considered statistically significant. The data analysis was performed using SPSS version 19 (SPSS Inc., Chicago, IL, USA). The study was conducted according to the good clinical practice guideline as well as the Declaration of Helsinki and was approved by our local ethics committee. All subjects signed informed consent to participate in this study.

Results

Among 329 dyspeptic Lao patients, 119 patients (36.2%) including 62 males and 57 females had *H. pylori* infection. The mean age of *H. pylori* positive patients was 46 years. There were 17 ethnic highland Lao (Lao Sung) (14.3%), 6 ethnic midland Lao (Lao Thueng) (5%), and 96 ethnic lowland Lao (Lao Lum) (80.7%). Total of 119 cases (83 from patients with gastritis, 13 with GU, 20

with DU and 3 with gastric cancer) were included in the analysis of this study.

Relationship between H. pylori virulence factors and clinical outcomes

Among 119 *H. pylori* positive patients, 118 patients had positive for *cagA* gene (99.2%) (Table 1). The majority of *cagA* genotype was East-Asian-type *cagA* (74/118, 62.7 %), and Western type *cagA* was found only 37.3 % (44/118). The East-Asian-type *cagA* was more commonly found in gastric ulcer (66.7%) than duodenal ulcer patients (50%) but the differences could not reach statistically significant (Table 1). Although, there were only 3 patients with gastric cancer but all showed East-Asian-type *cagA*.

In *vacA* s region, Almost all patients had s1 genotype (99.2%); s1c genotype was predominant (64.7%) followed by s1a (33.6%) and s1b (0.8%) genotype. As for *vacA* m region, m2 was predominant (56.3%) genotype followed by m1b (42%); whereas m1a and m1c genotypes were found only 0.8% (Table 1). There were no relationship between *vacA* s region and clinical outcomes. In contrast, the prevalence of m1b was significantly higher in gastric ulcer than chronic gastritis patients (69.2% *vs*. 35%, p=0.04). Accordingly, the prevalence of m2 was significantly lower in gastric ulcer compared with chronic gastritis patients (30.8% *vs*. 57.8%, p=0.04). When we combined the vagA s and m genotypes, the prevalence of s1a-m2 genotype was significantly higher in duodenal ulcer compared with chronic gastritis patients (40.0% *vs*. 16.9%, p=0.03). The prevalence of s1c-m1b genotype was significantly higher in gastric ulcer compared with chronic gastritis patients (53.8% *vs*. 24.1%, p=0.04). On the other hand, the prevalence of s1c-m2 genotype was significant higher in chronic gastritis than gastric ulcer patients (42.2% *vs*. 7.7%, p=0.02) as shown in Table 2.

Relationship between H. pylori virulence factors and ethnic group

All 17 ethnic highland Lao were infected with *H. pylori* possessing East-Asian-type *cagA* (Table 2). On the other hand, East-Asian-type *cagA* and Western-type *cagA* were found in ethnic lowland Lao as 55.8% and 44.2%, respectively. The prevalence of East-Asian-type *cagA* was significantly higher in ethnic highland Lao than that of lowland Lao (p=0.001). Among 6 cases from mildland Lao, 4 possessed East-Asian type *cagA* and 2 were Western type *cagA*. Overall, the prevalence of East-Asian-*cagA* was varies, decreasing at increasingly southerly latitudes. Majority of *vacA* s genotype in ethnic highland Lao were s1c and significantly higher than lowland Lao

Table 1. Virulence Factors of Lao *H. pylori* and Clinical Outcomes

		Total N= 119	Chronic gastritis N= 83	Gastric ulcer N= 13	Duodenal ulcer N= 20	Gastric cancer N= 3
cagA positive						
	East Asian type	74 (62.2%)	53 (63.9%)	8 (66.7%)	10 (50%)	3 (100%)
	Western type	44 (37%)	30 (36.1%)	4 (33.3%)	10 (50%)	0 (0%)
	cagA negative	1 (0.9%)				
vacA s						
	s1a	40 (33.6%)	26 (31.3%)	4 (30.8%)	10 (50%)	0 (0%)
	s1b	1 (0.8%)	0 (0%)	1 (7.7%)	0 (0%)	0 (0%)
	s1c	77 (64.7%)	56 (67.5%)	8 (61.5%)	10 (50%)	3 (100%)
	s2	1 (0.8%)	1 (1.2%)	0 (0%)	0 (0%)	0 (0%)
vacA m						
	m1a	1 (0.8%)	1 (1.2%)	0 (0%)	0 (0%)	0 (0%)
	m1b	50 (42%)	33 (39.8%)	9 (69.2%)*	7 (35%)	1 (33.3%)
	m1c	1 (0.8%)	1 (1.2%)	0 (0%)	0 (0%)	0 (0%)
	m2	67 (56.3%)	48 (57.8%)	4 (30.8%)*	13 (65%)	2 (66.7%)
Combine						
	s1a-m1b	15 (12.6)	12 (14.5%)	1 (7.7%)	2 (10%)	0 (0%)
	s1a-m2	25 (21%)	14 (16.9%)	3 (23.1%)	8 (40%)*	0 (0%)
	s1c-m1b	33 (27.7%)	20 (24.1%)	7 (53.8%)*	5 (25%)	1 (33.3%)
	s1c-m2	43 (36.1%)	35 (42.2%)**	1 (7.7%)	5 (25%)	2 (66.7%)

*P value <0.05 when compared with chronic gastritis; ** P value < 0.05 when compared with gastric ulcer

Table 2. Virulence Factors of Lao *H. pylori* and Ethnic Group

		Total N= 119	highland Lao N= 17	midland Lao N= 6	lowland Lao N= 96
cagA positive					
	East Asian type	74 (62.2%)	17 (100%)*	4 (66.7%)	53 (55.8%)
	Western type	44 (37%)	0 (0%)	2 (33.3%)	42 (44.2%)**
	cagA negative	1 (0.9%)	0 (0%)	0 (0%)	0 (0%)
vacA s					
	s1a	40 (33.6%)	2 (11.8%)***	3 (50%)	35 (36.5%)
	s1b	1 (0.8%)	0 (0%)	0 (0%)	1 (1%)
	s1c	77 (64.7%)	15 (88.2%)***	3 (50%)	59 (61.5%)
	s2	1 (0.8%)	0 (0%)	0 (0%)	1 (1%)
vacA m					
	m1a	1 (0.8%)	0 (0%)	0 (0%)	1 (1%)
	m1b	50 (42%)	7 (41.2%)	5 (83.3%)	38 (39.6%)
	m1c	1 (0.8%)	0 (0%)	0 (0%)	1 (1%)
	m2	67 (56.3%)	10 (58.8%)	1 (16.7%)	56 (58.3%)

*P value =0.001 when compared with lowland Lao; **P value=0.001 when compared with highland Lao; ***P value < 0.05 when compared with lowland Lao

(88.2% *vs*. 61.5%, p=0.03). In contrast, s1a genotype was significantly lower in ethnic highland Lao compared with ethnic lowland Lao (11.8% *vs*. 36.5%, p=0.04). Among 6 cases from mildland Lao, s1a and s1c were found in 3 and 3 cases, respectively. As for *vacA* m region, m2 is predominant in highland (58.8%) and lowland (58.3%) Lao. Only 1 case in midland Lao showed m2 genotype. In contrast, m1b is predominant in midland Lao. However, the differences were not statistically significance.

Discussion

H. pylori cagA-positive strains have been reported to be associated with severe diseases such as atrophic gastritis and gastric cancer (Hamlet et al., 1999; Andreson et al., 2002; Podzorski et al., 2003). In this study, *cagA* gene was found in nearly all patients (99.2%) and the majority of *cagA* genes were East-Asian-type (62.7%). This prevalence is similar to the previous studies that have shown positive *cagA* gene more than 90% in Asian countries, and had no association with disease outcome (Maeda et al., 1998; Kim et al., 2001; Zhou et al., 2004; Nguyen et al., 2011). The East-Asian-type *cagA* strain was sometimes considered to be more virulent than Western-type *cagA* strain (Azuma et al., 2004; Vilaichone et al., 2004). Interestingly, all 3 cases of gastric cancers in this study were East-Asian-type *cagA*. The possible explanation might be due to small number of gastric cancer patients. A large future study of gastric cancer patients in Lao might be performed to identify this relationship.

The s1/m1 strains are reported to be more virulence than s1/m2 and s2/m2 (Atherton et al., 1995). Compatible with the prior study, our study demonstrated that m1b was significantly higher in gastric ulcer than those in chronic gastritis and m2 was significantly lower in gastric ulcer than chronic gastritis. When we combined *vacA* s and m regions together, we found that s1c-m1b were significantly associated with gastric ulcer which had similar result to Taiwan study (Wang et al., 1998).

In Laos there are 3 major ethnic groups, ethnic highland Lao who live in North and East part that have been sharing the border with China and Vietnam. Lowland Lao who live in Central and South part and share the border with Thailand and Cambodia. These findings might explain that East-Asian-type *cagA* type was demonstrated in all strain from highland Lao as similar with China and Vietnam (Zhou et al., 2004; Nguyen et al., 2011; Uchida et al., 2009), and Western-type *cagA* which mainly found in ethnic lowland were similar to Thailand and Cambodia (Vilaichone et al., 2004; Breurec et al., 2011). For the same explanation, majority of *vacA* subtype were s1c, and m2 which found in strains from ethnic highland and similar to China and Vietnam (Zhou et al., 2004; Uchida et al., 2009; Nguyen et al., 2011), whereas *vacA* s1a and m2 genes were commonly found in lowland Lao which similar to Thailand and Cambodia (Vilaichone et al., 2004, Breurec et al., 2011) as sharing border together.

In conclusion *H. pylori* was a common infection in Laos same as other countries in Southeast Asia. *cagA* gene was demonstrated in nearly all Laos patients. Interestingly, *cagA* and *vacA* genotype might be important factors to explain *H. pylori* infection and disease outcomes in Laos.

The great diversity of *H. pylori* genotype in Laos suggests that future examination of *H. pylori* from different area and ethnic groups of other Southeast Asian countries as well as in the surrounding regions will provide new evidences both in diversity of *H. pylori* and migration of human hosts.

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