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Application of *vac*A Sequencing in *Helicobacter pylori* for Classification of Specimens from Healthy Persons, and from Hepatobiliary and Gastroduodenal Patients

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***Helicobacter pylori* is an important causative agent of gastrointestinal and hepatobiliary diseases. The aim of this study was to investigate phylogenetic relationships among *H. pylori* strains from the oral cavities of healthy individuals, gastric biopsies from gastroduodenal (GI) patients and bile samples from hepatobiliary (HB) patients in the northeast of Thailand. The DNA sequences of a portion of the vacuolating cytotoxin A gene (*vac*A) were investigated. Phylogenetic trees were constructed using the neighbor-joining and maximum-likelihood methods. The *vac*A sequences of *H. pylori* fell into four main groups on the trees. Strains from healthy persons fell into two widely separated but well-supported groups, while most *H. pylori vac*A sequences from HB patients were distributed in another two groups. In contrast, the *H. pylori* strains from GI patients were scattered across the tree, without a clear geographical pattern. In conclusion, the sequence of *vac*A may be useful to classify the genetic relationship of *H. pylori* derived from different sources.**

Keywords: *H. pylori*, vacuolating cytotoxin A gene, Gastrointestinal diseases, hepatobiliary diseases and healthy persons.

Helicobacter pylori is a Gram-negative spiral-shaped bacterium, a causative agent of many gastrointestinal diseases such as dyspepsia, gastritis, peptic ulcer and gastro-esophageal reflux disease, which can lead to gastric adenocarcinoma[1]. This pathogen has also been recognized as a type I carcinogen by the World Health Organization's International Agency for Research on Cancer because it is associated with gastric mucosa-associated lymphoid tissue lymphoma and gastric adenocarcinoma[2].

Helicobacter pylori was recently proposed to be associated with diseases outside the gastroduodenal tract, particularly the hepatobiliary duct system[3]. Such diseases include cholangiocarcinoma (CCA), which is a primary cancer of the biliary epithelium and is highly endemic in Northeast Thailand[4]. Several studies have demonstrated that the virulence genes in *H. pylori*, including the vacuolating cytotoxin gene A (*vac*A) and cytotoxin-associated gene A (*cag*A),

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may help to predict the association between *H. pylori* infection and clinical outcomes[4,5]. However, *cag*A and other virulence genes (viz. *bab*A, *cag*E and *ice*A) are present in only some strains of *H. pylori*, whereas *vac*A is present in all *H. pylori* strains[6].

Vacuolating cytotoxin induces intracellular vacuoles, leading to epithelial damage of eukaryotic cells[7] by suppressing epithelial proliferation, promoting apoptotic cell death[8], inducing cytoskeletal changes[8] and suppressing epithelial proliferation[9]. Each allelic variant of *vac*A contains one of two classes of signal-region variant (s1 or s2) and one of two classes of middle region (m1 or m2) variant[10]. Allelic variation among strains results in variation of vacuolating activity[11]. The s1/m1 *H. pylori* strain displays a higher vacuolating activity than that does the s1/m2 strain and might be associated with gastritis and gastric adenocarcinoma. In contrast, the *vac*A s2/m2 strain is rarely associated with such diseases due to the absence of cytotoxic activity[12]. Although, *vac*A genotype status might help predict the development of diseases, one report has suggested that genotype of *vac*A was not associated with the clinical outcome of peptic ulcer, duodenal ulcer and gastric cancer[13]. Therefore, in this study, we test the idea that the *vac*A sequence can be used to distinguish among clinical sources of specimens.

Routes of *H. pylori* transmission are oral-oral[14], gastro-oral[15] and fecal-oral[16]. Saliva can be a reservoir of *H. pylori*, potentially infecting or reinfecting the stomach[17]. It might be that the genotypes of *H. pylori* strains in saliva of healthy persons can be useful for mapping the geographical distribution of *H. pylori* strains associated with gastroduodenal (GI) and hepatobiliary (HB) diseases. To explore this possibility, we sequenced portions of the *vac*A gene from specimens from healthy individuals and from patients with various gastroduodenal and hepatobiliary diseases.

MATERIALS AND METHODS

Study subjects

Study subjects consisted of healthy persons, patients with GI diseases and patients with hepatobiliary diseases admitted to Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, Thailand. Approval for the study was obtained from the Human Ethics Committee of Khon Kaen University (approval nos. HE571489 and HE581271) and prior written informed consent was obtained from all participants.

Collection and processing of saliva samples

Thirty saliva samples were collected from healthy person using the method of Silva *et.al* (2009), with slight modifications[18]. Two ml of each saliva sample was added to 8 ml of Brucella broth (CRITERION™, USA) and incubated for 3 days at 37 °C with shaking under microaerobic atmosphere (5% O_2, 10% CO_2 and 85% N_2). After centrifugation at 13,000 rpm for 10 minutes at 4 °C, the pellet from each culture was used as a source of DNA for amplification of *vac*A by nested PCR.

Collection and processing of gastric biopsy and bile specimens

Gastric biopsies were performed on dyspeptic patients undergoing gastro-endoscopic examinations at the Endoscopy Unit, Srinagarind Hospital, Faculty of Medicine, Khon Kaen University. Dyspeptic patients (GI) were characterized as having non-ulcer disease (NUD) (n = 20), peptic ulcer disease (PUD) (n = 3) or gastric cancer (GC) (n = 2). Gastric samples from these patients were positive for the rapid urease test indicating *H. pylori* infection. Bile samples, positive for *H. pylori* via PCR detection of *ure*A gene, were taken from patients with hepatobiliary disorders, namely, CCA (n = 20) and cholelithiasis (n = 9), at the Surgical Unit, Srinagarind Hospital, Faculty of Medicine, Khon Kaen University. The sources of the clinical specimens are summarized in Table 1.

DNA extraction

DNA was extracted from pellets of saliva samples using a modified method based on the Puregene DNA Purification System (Gentra System, USA)[19]. DNA was extracted from bile samples and biopsy specimens using the Gentra System DNA extraction and purification kit (Big Lake, MN, USA), respectively, as previously described[4,20].

PCR amplification and DNA Sequencing

Amplification of a portion of the *vac*A gene from each sample was performed by nested PCR using primers and conditions as shown in Table 2. The inner primers (forward: nt. 2673-2692, reverse: nt. 2929-2948; GenBank GQ331975) amplified a fragment of *vac*A encoding amino acid positions 892 to 979. The region amplified does

not include the s or the m region. Nested PCR assay was performed in a total volume of 25 µl containing 500 ng of DNA template, 0.2 mM dNTPs (Amresco, Ohio, USA), 1X PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.1 mg/ml bovine serum albumin, 10 mM $(NH_4)_2SO_4$, and 1.5 mM $MgCl_2$) (RBC bioscience, Taipei, Taiwan), 0.5 U *Taq* polymerase (RBC Bioscience, Taipei, Taiwan), and 0.4 µM *vac*A primers. Thermocycling was conducted in a C1000™ Thermal Cycler (BioRad, USA). Amplicons were purified using spin column PCR clean up kit (DNA sequencing service; BIONEER, Korea) and sequenced using Sanger sequencing method (DNA sequencing service; BIONEER, Korea).

Phylogenetic analysis

The *vac*A sequences from all samples, including sixteen reference sequences of *H. pylori vac*A from GenBank (Table 1), were aligned using the MAFFT program (http://www.ebi.ac.uk/Tools/msa/mafft). The aligned sequences were adjusted and edited using BioEdit v.7.0.5.2. Phylogenetic analyses were conducted in MEGA software version 7.0[21]. The phylogenetic trees were constructed and compared using the neighbor-joining and maximum-likelihood methods with 1,000 bootstrap replicates. The Kimura 2-parameter model with Gamma distribution was used as the best model (the model giving the best log-likelihood value.

RESULTS

The phylogenetic trees (Figs. 1 and 2) revealed that most sequences from Thai samples fell into distinct clades and were generally also distinct from sequences from other countries. The alignment of 101 partial sequences of the *vac*A gene was 276 bp in length, and translated to 88 amino acids (Fig. 3).

The neighbor-joining tree (Fig. 1) placed the thirty sequences from healthy Thai people into two very distinct groups with high bootstrap support: group 1 (82% support) consisted of 23 sequences and group 4 (97% support) included 6 sequences. Sequences from Thai hepatobiliary patients mostly fell into two groups, group 2 consisting of 10 sequences from CCA patients together with 5 sequences from Thai gall-stone patients (58% bootstrap support), and group 3 consisting of 8 sequences from CCA patients and 1 sequence from patients with other hepatobiliary

Table 1. Sources of samples used in this study

Source of samples	Number of samples	Sample code
Healthy persons (n=30)		
• Saliva samples	30	-
Gastroduodenal patients (n=25)		
• Non-ulcer disease (NUD)	20	-
• Peptic ulcer diseases (PUD)	3	-
• Gastric cancer (GC)	2	-
Hepatobiliary patients (n=30)		
• Cholangiocarcinoma (CCA)	20	-
• Cholelithiasis	9	-
• Other hepatobiliary diseases	1	-
Reference strains*		
Japan	5	CP06826, AB190972, AB190973, AF071097, AB190965
India	3	GQ331975, GQ331980, GQ331984
Kenya	3	AF191641, AF191642, AF191644
USA	2	CP003474, AE000511
China	1	CP003419
Italy	1	U95971
West Africa	1	CP002571

* = All reference strains taken from Genbank were isolated from GI patients

diseases (98% support). In contrast, most *vac*A sequences from Thai GI patients were not close to those from healthy individuals or HB patients, but were scattered across the tree and often among *vac*A sequences of GI patients from various countries. Similarly, *H. pylori* sequences from saliva samples of healthy persons, gastric biopsies of gastrointestinal tract patients (GI) and bile samples of hepatobiliary patients were grouped in the maximum likelihood tree (Fig. 2) in a manner consistent with the neighbor joining tree. Bootstrap values differed slightly between the trees and the 4 groups, while still strongly supported, fell in somewhat different parts of the two trees.

In this study, we also determined the alleles of *vac*A (s and m) present in the bile samples of hepatobiliary patients which are nearly all of were s1 and m1 (data not shown). Therefore, these alleles can't be used to identify the different clinical origins of samples.

Translated amino acid sequences were compared among the four groups of *H. pylori* (indicated in Fig. 1 and 2) and found to differ at many positions (Fig. 3).

DISCUSSION

Our study is the first investigation on the genetic diversity and relationships of *vac*A gene sequences of *H. pylori* from different sources; saliva from healthy persons, gastric biopsies from gastrointestinal tract patients (GI) and bile samples from hepatobiliary patients (CCA and cholelithiasis) in Thailand. Partial *vac*A gene sequences from healthy persons were obviously very different from those of HB patients. *H. pylori* strains from saliva samples of healthy persons fell into two well-supported but widely separated clades (clades 1 and 4). Strains isolated from HB patients, especially those with CCA, mostly also fell into distinct clades (clades 2 and 3). These results indicate that particular genotypes of *vac*A may occur in strains of *H. pylori* specific to particular sources (disease status of the host and geography). This applied strongly to strains from CCA patients: this disease is highly endemic in Thailand. Interestingly, most *H. pylori* strains from GI tracts of Thai patients were distributed among other strains of GI patients from different countries, but not among the strains from healthy persons or HB patients. It seems that most *H. pylori* strains in saliva of healthy persons in Northeast Thailand may not be derived from the strains isolated from GI and HB patients. The partial *vac*A gene sequence developed in this study has the potential to distinguish among *H. pylori* strains from different origins and sources. In this study, genetic diversity and relationships of *H. pylori* from different sources were determined using neighbor-joining and maximum-likelihood methods. Results from the two methods were in broad agreement, suggesting we were close to the true phylogenetic tree. An advantage of the neighbor-joining method is more computational efficiency and speed than maximum-likelihood method, making it suitable for analysis of large data sets. However, this method is unsuitable if very divergent sequences are included[22]. The maximum-likelihood (ML) approach is character-based and is now widely used owing to the development of increasingly realistic and explicit models of sequence evolution. However, likelihood calculations and tree searches under the likelihood criterion are computationally

Table 2. Primer sequences and PCR condition for detection of *H. pylori*

Specific for	Primer sequence (5'>>>3')	Product size (bp)	PCR condition
*vac*A[19]	OF-GCATGATTTTGGCACCATTG	429	95 °C 30 s, 54 °C 30 s,
(*H. pylori*)	OR-TTTTCATATTTAGGGGCAAA		72 °C 45 s (35 cycles)
	IF-GCATGATTTTGGCACCATTG	276	95 °C 30 s, 62 °C 30 s,
	IR-ATCGCATTGCTCAAGCTCAA		72 °C 45 s (35 cycles)
*vac*A s1/s2[24]	F-ATGGAAATACAACAAACACAC	259/286	94 °C 60 s, 58 °C 60 s,
	R-CTGCTTGAATGCGCCAAAC		72 °C 60 s (35 cycles)
*vac*A m1/m2[24]	F-CAATCTGTCCAATCAAGCGAG	567/642	94 °C 60 s, 55 °C 60 s,
	R-GCGTCAAAATAATTCCAAGG		72 °C 60 s (35 cycles)

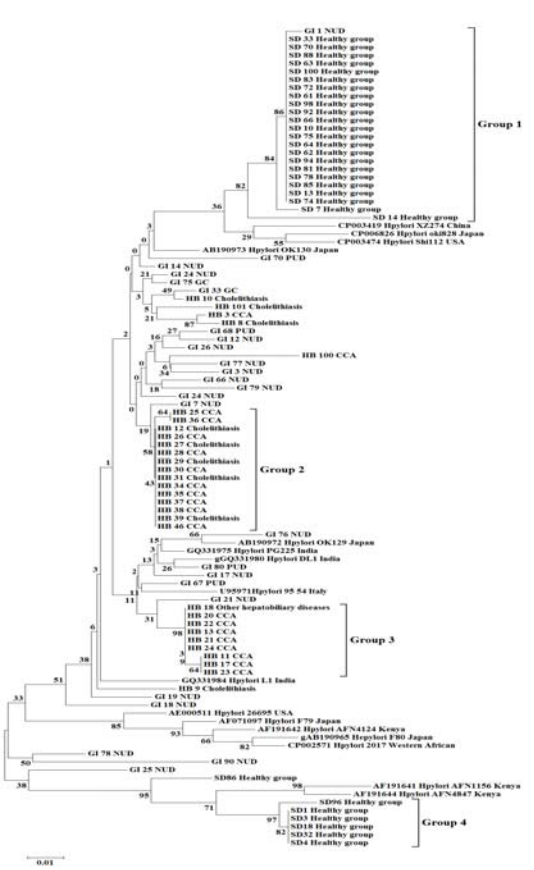

Fig. 1. Neighbor joining tree with 1,000 bootstraps from a dataset of partial *vac*A gene sequences. Numbers on the branches are bootstrap values (%). Disease conditions are represented by NUD; nonpeptic ulcer diseases, PUD; peptic ulcer diseases and CCA; cholangiocarcinoma

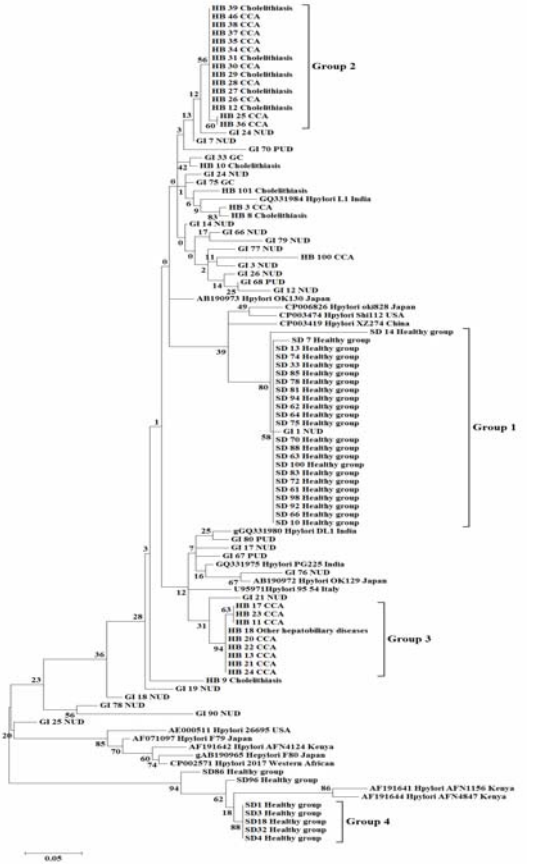

Fig. 2. Maximum likelihood tree with 1,000 bootstraps from a dataset of partial *vac*A gene sequences. Numbers on the branches are bootstrap values (%). Disease conditions are represented by NUD; nonpeptic ulcer diseases, PUD; peptic ulcer diseases and CCA; cholangiocarcinoma

Fig. 3. Differences among the source-groups of *H. pylori* strains in an 88-amino acid portion translated from the *vac*A gene sequence. *H. pylori* PG225 is the reference strain from the NCBI database (GQ331975). Abbreviation of amino acids represented by A, Alanine; C, Cysteine; D, Aspartic acid; E, Glutamic acid; G, Glycine; H, Histidine; I, Isoleucine; K, Lysine; L, Leucine; N, Asparagine; Q, Glutamine; R, Arginine; S, Serine; T, Threonine; V, Valine

demanding, making this approach very slow, especially if many sequences are included in the alignment[22]. Researchers can choose the method that best suits their data[24].

Differences were apparent between the four clades of sequences at both the DNA and the amino acid level. Presumably, this is a consequence of adaptation to different environmental sources, hosts and geography[13,23]. However, the relationship between *vac*A diversity, origin, and disease state in the host should be further investigated.

In conclusion, these findings support that the partial *vac*A sequence may be useful as a marker to distinguish *H. pylori* from difference sources, such as from healthy persons or patients with gastrointestinal and hepatobiliary diseases, and as a marker to investigate genetic relationships among the strains isolated from different sources.

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