CHAPTER IV

MATERIALS AND METHODS

1. Patients and *H. pylori* isolates

A total of 360 patients presenting upper gastrointestinal symptoms were enrolled in the study and all patients gave informed consent. All patients had not been submitted to eradication in the previous 3 mounts. Specimens were obtained from antral biopsies of patients attending Chulalongkorn hospital between August 2003 to June 2004. Two biopsies were kept in 0.85% normal saline, transported cold to the laboratory and culture immediately. Endoscopic findings were recorded and according to endoscopic findings, patients were divided into two groups: Group 1, Patients with peptic ulcer ; Group 2, Patients with non-ulcer dyspepsia (NUD). There were 292 patients with NUD, 57 patients with gastric ulcer and 11 patients with duodenal ulcer. In addition to clinical specimens, 18 *H. pylori* clinical isolates maintained at -70°C were included in this study. Fifteen were from patients with gastric ulcer, two were from patients with NUD and one was from patients with duodenal ulcer.

DNA marker for type(s) and subtype(s) of genes *cagA, vacA* and *iceA* of *H. pylori* were kindly provided by Dr. Yoshio Yamaoka, Department of Medicine, Baylor College of Medicine, Houston, Texas, U.S.A.

2. Culture and identification

Gastric biopsy specimens were inoculated on both selective medium columbia agar with antibiotics (containing vancomycin, amphotericin B, cefsoludin and trimethoprim) and without antibiotics containing 7% sheep blood. The plates were incubated at 35°C to 37°C for 3 to 7 days in an anaerobic jar (BBL Microbiology System, Cockeysville, Md.) under microaerobic atmosphere.

The plates were visually inspected for small, translucent and glistening with a convex elevation and an entire edge colonies. Suspected colonies were subjected to definitive identification by biochemical reaction tests as follows :

2.1 Urease test

A. Inoculum

Active growing colonies from solid media

B. Reagent

Urea test broth and store at 4°C

C. Procedure

Five hundred microliters of the urease test broth were dispensed into 1.5 ml microcentrifuge tube. A heavy inoculum of colonies from 3 - 4 days old plate with the typical morphologic characteristic of *H. pylori* was inoculated into the broth. The suspension was mixed thoroughly and observed for color development.

D. Results

Development of a color change within 5 minutes is indicative of a positive result. Any color development after 5 minutes is reported as a negative test for urease.

2.2 Catalase test

A. Inoculum

Active growing colonies from solid media

B. Reagent

- Hydrogen peroxide (3 %) stored at room temperature
- Sterile loop
- Clean glass slide

C. Procedure

With a sterile loop, a small amount of bacterial culture from the agar was transferred onto the surface of a clean, dry glass slide. A drop of 3 % hydrogen peroxide was

immediately placed onto a portion of the culture. The production of bubbles of gas was observed.

D. Results

Catalase positive organism will produce bubbles upon addition of the hydrogen peroxide, Catalase negative organism will not produce any visible bubbles.

2.3 Oxidase test

A. Inoculum

Active growing colonies from solid media.

B. Reagent

Oxidase reagent : (tetramethy -*p*-phenylenediamine dihydrochloride)

C. Procedure

A portion of the colony or culture growth to be tested was picked up and rubbed directly onto a portion of a reaction area of the dry slide oxidase.

The reaction area was examined for the appearance of a dark purple color within 20 seconds.

D. Result

Oxidase - positive organisms produce a purple or dark purple color within 20 seconds. Oxidase - negative organisms produce no color change within 20 seconds.

3. DNA extraction

The DNA was purified according to the manufactor's direction by using the QIA amp® DNA Mini Kit (Qiagen Corporation, Germany). Briefly, bacteria were removed from culture plate with an inoculation loop. Bacterial cell lysis was performed by adding 180 µl of ATL buffer and 20 µl of proteinase K to the cell pellet. After well mixing and the suspension was incubated at 56°C until the pellet was completely lysed, 200 µl of the buffer AL were added. The suspension was heated at 70°C for 10 min and 200 µl ethanol were added. The suspension was then transferred into QIAamp spin column in a clean 2 ml collection tube,

centrifuged at 8,000 rpm for 1 min and the tube containing the filtrate was discarded. QIAamp spin column was then placed in a clean 2 ml collection tube, opened and 500µl buffer AW1 were added. It was then centrifuged at 8,000 rpm for 1 min and the tube containing the filtrate was discarded. QIAamp spin column was then washed with buffer AW2 and centrifuged at 14,000 rpm for 3 min. After centrifugation the QIAamp spin column was placed in a clean 1.5 ml microcentrifuge tube and 200 µl buffer AE were added and the incubated at room temperature for 1 min. After incubation, the tube was centrifuged at 8,000 rpm for 1 min and eluted buffer was stored at -20°C, ready for the PCR analysis.

4. Genotyping methodology

4.1 Polymerase chain reaction for genes *cagA*, *vacA* and *ice A*

Polymerase chain reaction was performed by the method of Yamaoka, et al. (35) as seen in the flow chart (Figure 4). Diagram in Figure 5, Figure 6 and Figure 7 demonstrated the amplification by specific primers for *cagA*, *vacA* and *iceA*, respectively. In brief PCR reactions were performed in a volume of 25 µl containing 25 pmol of each primer (Table 7) 1X PCR buffer (10 mM Tris - HCl, 50 mM KCl), 2.5 mM $MgCl_2$, 200 µM of each of 4 dNTPs and 1U of Taq polymerase. PCR reaction was performed in the thermal cyeler (Perkin Elmer GeneAmp system 9600) under specific PCR condition that consisted of an initiation denaturation at 94°C for 10 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 52°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 7 min.

4.2 Detection of amplification product

Ten microlites of PCR product were mixed with 3 µl of gel loading buffer (20 % ficoll, 0.05 % bromophenol blue), analysed by electrophoresis in 2 % agarose gel containing 0.5 µg/ml ethidium brominds in 1X Tris-borate buffer (pH 8.0). The electrophoresis was carried out at 80 volts for 45 min. Gel was visualized under UV light by Camera Gel Doc™

experiment. A molecular ladder of 100 - bp was used to estimate the size of the PCR fragments.

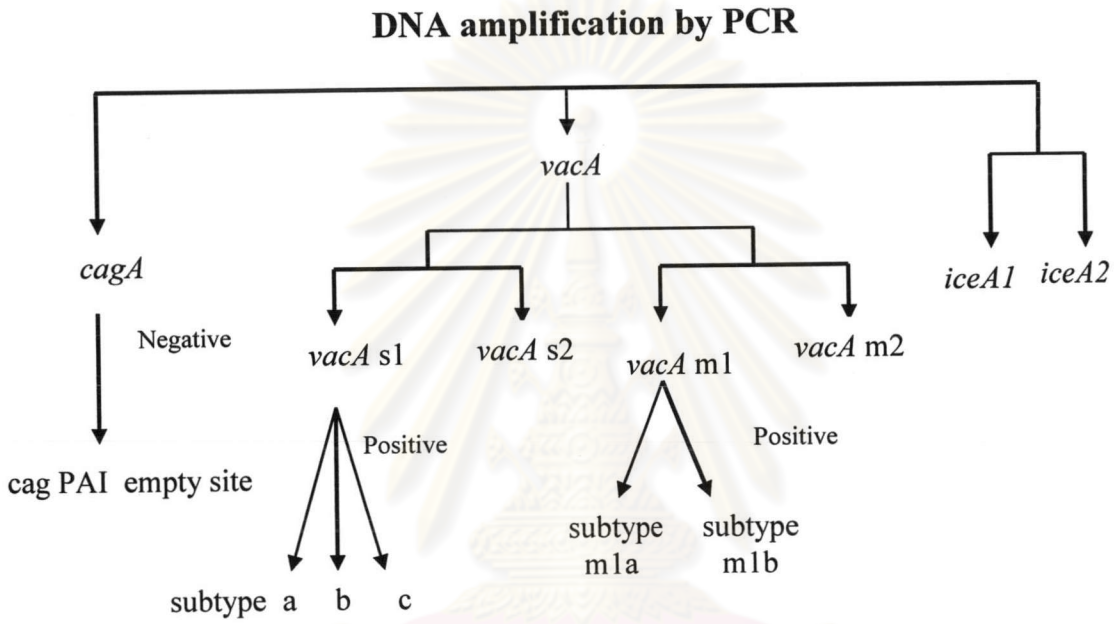

Figure 4. DNA amplification of genes *cagA, vacA* and *iceA* in *H. pylori*

***cagA* gene**

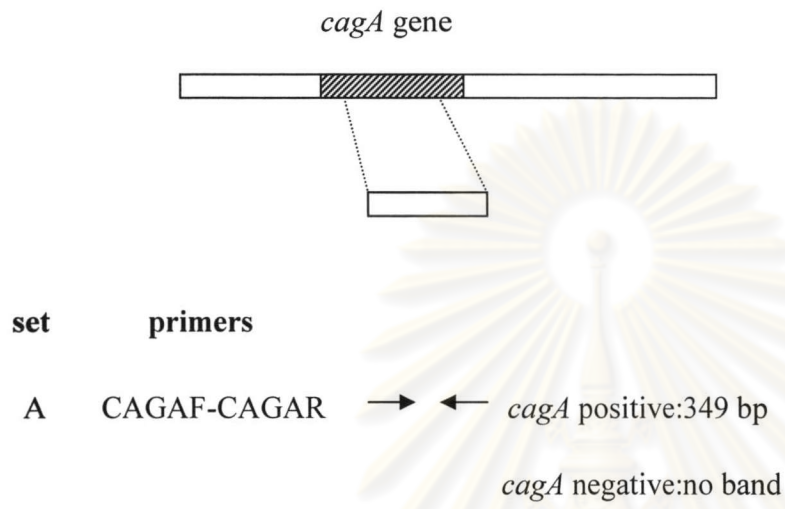

Figure 5. Diagram present target gene for amplification *cagA* gene

***vacA* gene**

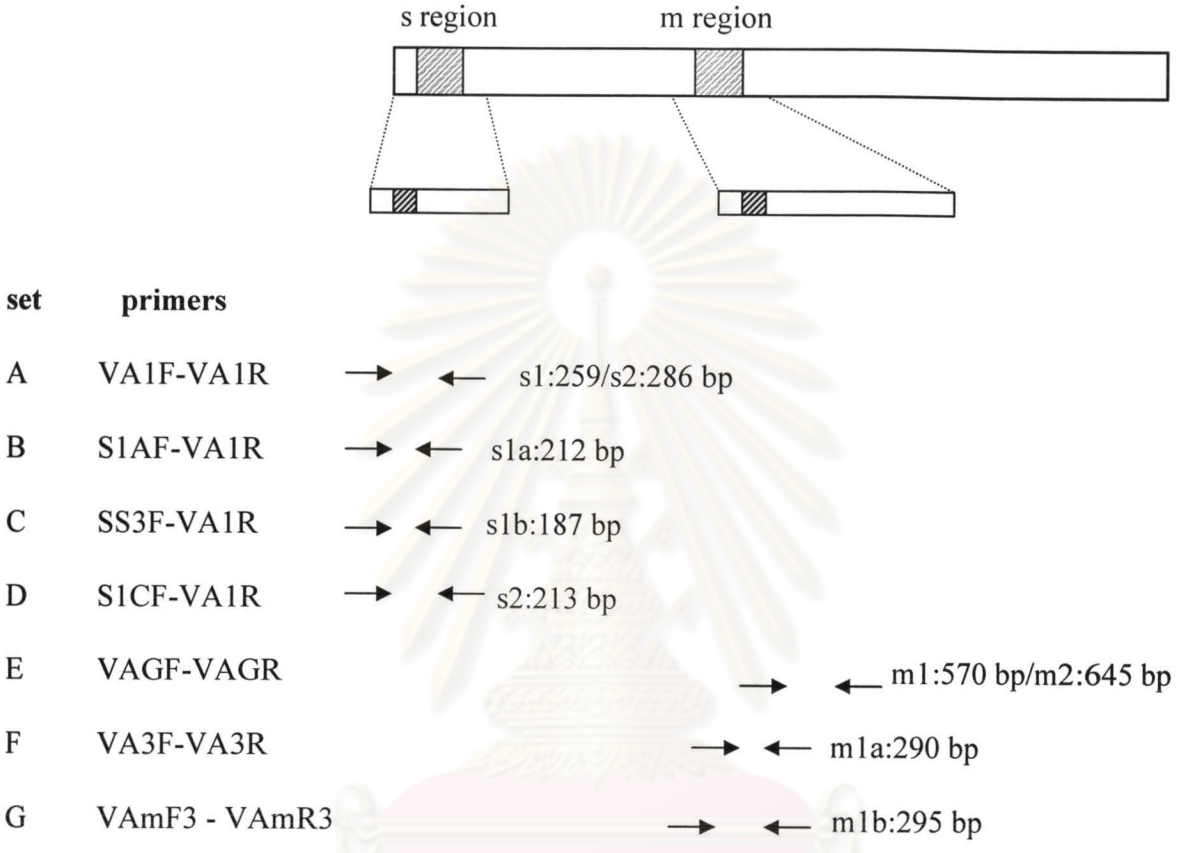

Figure 6. Mosaic structure of the *vacA* genes comprising the variable s and m regions

***iceA* gene**

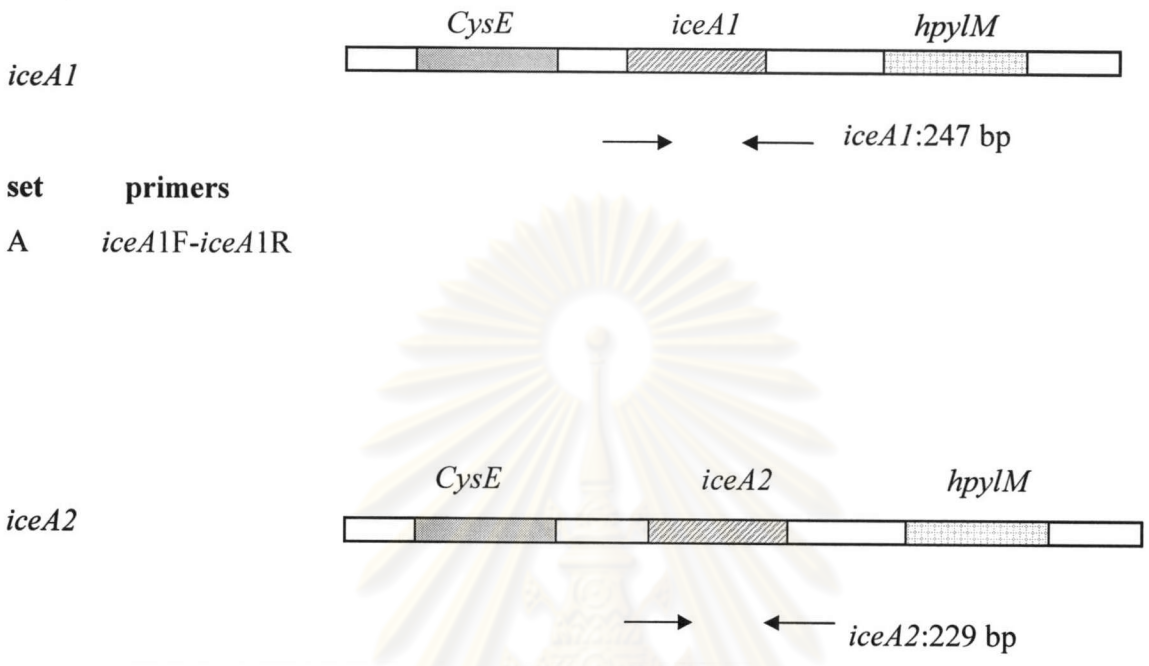

Figure 7. Diagram present target gene for amplification *iceA* gene

Table 7. PCR primers for amplification of genes *cagA, vacA* and *iceA* in *H. pylori*

Gene and DNA region amplified	Primer	Primer sequence	Size (bp) of PCR product (location)	Reference
cagA	CAGAF	GATAACAGGCAAGCTTTTGAGG	349 (1228-1576)[c]	35
	CAGAR	CTGCAAAAGATTGTTTGGCAGA		
cag PAI empty site	Luni 1	ACATTTTGGCTAAATAAACGCTG	550 [f]	184
	R5280	GGTTGCACGCATTTTCCCTTAATC		
vacA s1	VA1-F	ATGGAAATACAACAAACACAC	259 (797-1055)[d]	35
	VA1-R	CTGCTTGAATGCGCCAAAC		
vacA s2	VA1-F	ATGGAAATACAACAAACACAC	286 (284-569)[e]	35
	VA1-R	CTGCTTGAATGCGCCAAAC		
vacA s1a	S1A-F[b]	TCTYGCTTTAGTAGGAGC	212 (844-1055)[d]	35
vacA s1b	SS3-F[b]	AGCGCCATACCGCAAGAG	187 [f]	35
vacA s1c	S1C-F[b]	CTYGCTTTAGTRGGGYTA	213 [f]	35
vacA m1	VAG-F	CAATCTGTCCAATCAAGCGAG	570 (2071-2640)[d]	35
	VAG-R	GCGTCTAAATAATTCCAAGG		
vacA m2	VAG-F	CAATCTGTCCAATCAAGCGAG	645 (639-1283)[e]	35
	VAG-R	GCGTCTAAATAATTCCAAGG		
vacA m1a	VA3-F	GGTCAAAATGCGGTCATGG	290 [f]	184
	VA3-R	CCATTGGTACCTGTAGAAAC		
vacA m1b	VAm-F3	GGCCCCAATGCAGTCATGGAT	295 [f]	184
	VAm-R3	GCTGTTAGTGCCTAAAGAAGCAT		
iceA1	*iceA* 1F	GTGTTTTTAACCAAAGTATC	247 (857-1103)[g]	35
	iceA 1R	CTATAGCCASTYTCTTTGCA		
iceA2	*iceA* 2F	GTTGGGTATATCACAATTTAT	229 (283-604)[h]	35
	iceA 2R	TTRCCCTATTTTCTAGTAGGT		

[a] Y is C or T, M is A or C, S is C or G, and R is A or G

[b] Used with primer VA1-R

[c] Nucleotide positions in the *cagA* gene of *H. pylori* ATCC 53726 (GenBank accession no. L117714)

[d] Nucleotide positions in the *vacA* gene of *H. pylori* 60190 (GenBank accession no. U05676)

[e] Nucleotide positions in the *vacA* gene of *H. pylori* Tx30a (GenBank accession no. U29401)

[f] No published coordinates for genes in strains of these types

[g] Nucleotide positions in the *iceA*1 gene of *H. pylori* 60190 (GenBank accession no. U43917)

[h] Nucleotide positions in the *iceA*2 gene of *H. pylori* 213 (GenBank accession no. AF008928)

5. Statistical analysis

Fisher's exact test or chi square (X^2) test was used for analysis of categorical data. A multiple logistic regression analysis was performed using *H. pylori* genotypes, age and sex of the patients. Associations between categorical variables were determined using odds ration (OR) and 95 % confidence interval (95 % CI) were calculated using the statistical program SigmaStat for Windows version 3.1. A P value ≤ 0.05 was considered to be statistically significant.