การดื้อต่อยา amoxicillin ในเชื้อ *Helicobacter pylori* ซึ่งแยกได้จากผู้ป่วยไทย

นางสาว ปัญจพร ประเสริฐสิน

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AMOXICILLIN RESISTANCE IN *HELICOBACTER PYLORI* ISOLATED FROM THAI PATIENTS

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สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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สถาบันวิทยบริการ
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ปัญจพร ประเสริฐสิน: การดื้อต่อยา amoxicillin ในเชื้อ *Helicobacter pylori* ซึ่งแยกได้จากผู้ป่วยไทย (AMOXICILLIN RESISTANCE IN *HELICOBACTER PYLORI* ISOLATED FROM THAI PATIENTS) อาจารย์ที่ปรึกษา : อ. ดร.ธนิษฐา ฉัตรสุวรรณ, 84 หน้า. ISBN 974-17-4802-7

Amoxicillin เป็นหนึ่งในยาปฏิชีวนะที่ใช้ในการรักษาโรคติดเชื้อ *Helicobacter pylori* ในปัจจุบันการดื้อต่อยา amoxicillin ของเชื้อ *H. pylori* มีแนวโน้มเพิ่มสูงขึ้นในหลายประเทศ การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อหาความชุกของการดื้อต่อยา amoxicillin ของเชื้อ *H. pylori* ที่แยกได้จากผู้ป่วยไทย โดยตรวจหาค่า MIC โดยวิธี E-test และศึกษากลไกการดื้อต่อยา amoxicillin ของเชื้อ *H. pylori* โดยตรวจหาการกลายพันธุ์ของยีน *pbp1* โดยการเพิ่มปริมาณ DNA ด้วยเทคนิค PCR และหาลำดับเบสของยีน *pbp1* โดยวิธี DNA Sequencing เชื้อที่ใช้ในการศึกษาครั้งนี้แยกได้จากตัวอย่างชิ้นเนื้อของผู้ป่วยที่มารับการตรวจส่องกล้องระบบทางเดินอาหาร ที่หน่วยระบบทางเดินอาหาร โรงพยาบาลจุฬาลงกรณ์ระหว่างเดือนสิงหาคม พ.ศ. 2546 ถึง เดือนมิถุนายน พ.ศ. 2547 จำนวน 357 คน สามารถเพาะแยกเชื้อ *H. pylori* ได้ 50 สายพันธุ์ (14%, 50/357) เมื่อทำการทดสอบหาค่า MIC ต่อยา amoxicillin ทุกสายพันธุ์ พบว่าเชื้อจำนวน 49 สายพันธุ์ (98%) ให้ผลไวต่อยา amoxicillin และเชื้อจำนวน 1 สายพันธุ์ (2%) (MIC 0.75 μg/ml) ให้ผลดื้อต่อยา amoxicillin ซึ่งค่า MIC_{50} มีค่า <0.016 μg/ml และค่า MIC_{90} มีค่า 0.016 μg/ml เมื่อทำการวิเคราะห์ลำดับนิวคลีโอไทด์ในยีน *pbp1* ของเชื้อสายพันธุ์ที่ดื้อต่อยา 1สายพันธุ์ (HP-1144) และสายพันธุ์ที่ไวต่อยา จำนวน 5 สายพันธุ์ โดยเปรียบเทียบกับ *H. pylori* 26695 พบว่าในสายพันธุ์ที่ไวต่อยาเกิดการเปลี่ยนแปลง amino acid ใน PBP1 42 ตำแหน่งได้แก่ 13, 16, 17, 35, 45, 79, 114, 120, 125, 148, 242, 243, 322, 324, 332, 352, 374, 392, 406, 408, 414, 432, 469, 479, 504, 508, 509, 515, 534, 535, 543, 547, 556, 589, 593, 595, 611, 648, 649, 653, 654 , 656 และมี amino acid ขาดหายไปหนึ่งตำแหน่ง ส่วนสายพันธุ์ที่ดื้อต่อยาพบการเปลี่ยนแปลง amino acid 15 ตำแหน่ง ได้แก่ 17, 35, 79,125,148, 324,479, 504, 508, 509, 515, 535, 543, 648 และ 649โดยทั้ง 15 ตำแหน่ง ที่พบในสายพันธุ์ที่ดื้อต่อยานั้นเป็นตำแหน่งที่พบการเปลี่ยนแปลงในสายพันธุ์ที่ไวต่อยา นอกจากนี้ยังพบว่าการเปลี่ยนแปลงของ amino acid ใน PBP1 ในตำแหน่งที่ 406, 414, 515, 535, 543, 556, 593, 648, 649, และ 656 ที่มีผู้รายงานว่ามีความสัมพันธ์กับการดื้อยา amoxicillin นั้น พบได้ในสายพันธุ์ที่ไวต่อยา เมื่อทำการทดสอบเอนไซม์ beta-lactamase โดยวิธี chromogenic cephalosporin ใน HP-1144 พบว่าเชื้อไม่สร้างเอนไซม์ beta-lactamase ดังนั้นกลไกการดื้อต่อยา amoxicillin ใน HP-1144 อาจจะไม่มีความสัมพันธ์กับการเกิดการกลายพันธุ์ ในยีน *pbp1* และการสร้างเอนไซม์ beta-lactamase แต่อาจใช้กลไกอื่นๆ ได้แก่การลดการผ่านเข้าสู่เซลล์ของยา, การเปลี่ยนแปลง outer membrane protein และ efflux pump เป็นต้น

สาขาวิชาจุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต ปัญจพร ประเสริฐสิน

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Amoxicillin is an important component of combination therapies for *Helicobacter pylori* eradication and there is an increasing rate of amoxicillin resistance in many countries. The purpose of this study is to investigate the prevalence of amoxicillin resistance in *H. pylori* isolated from Thai patients and to determine the molecular mechanism of amoxicillin resistance in *H. pylori* by detecting mutations in the *pbp1* gene. The minimal inhibitory concentration (MIC) of amoxicillin was examined by the E-test. Mutations in the *pbp1* gene were analyzed by PCR and DNA sequencing. Fifty *H. pylori* isolates were obtained from 357 patients (14%, 50/357) who underwent upper gastrointestinal endoscopy at King Chulalongkorn Memorial hospital between August 2003 to June 2004. Of the 50 isolates, 49 (98%) were susceptible to amoxicillin and only one isolate was resistant (2%) (MIC 0.75 μg/ml). MIC_{50} and MIC_{90} were <0.016 and 0.016 μg/ml, respectively. The DNA sequences of *pbp1* were determined by sequencing both amoxicillin – susceptible and amoxicillin – resistant *H. pylori* isolates. Of the 6 isolates, 5 were susceptible to amoxicillin (MICs range = < 0.016 – 0.25 μg/ml) and one isolate, HP-1144, was resistant to amoxicillin (MIC = 0.75μg/ml). When compared with amino acid sequence of PBP1 of *H. pylori* 26695 from GenBank, the result from this study showed forty-two different amino acid substitutions in both amoxicillin-sensitive and resistant isolates at positions 13, 16, 17, 35, 45, 79, 114, 120, 125, 148, 242, 243, 322, 324, 332, 352, 374, 392, 406, 408, 414, 432, 469, 479, 504, 508, 509, 515, 534, 535, 543, 547, 556, 589, 593, 595, 611, 648, 649, 653, 654 , 656 and one amino acid deletion at position 400. Fifteen amino acid changes were shared among resistant and susceptible strains. All mutations in the resistant isolate, HP-1144, were found in susceptible isolates (positions 17, 35, 79,125,148, 324,479, 504, 508, 509, 515, 535, 543, 648 and 649). Interestingly, our results showed that mutations in PBP1 at position 406, 414, 515, 535, 543, 556, 593, 648, 649 and 656 which others investigators reported to be linked to amoxicillin resistance were present in our amoxicillin susceptible isolates. HP-1144 was tested for production of beta-lactamase using the chromogenic cephalosporin method and beta-lactamase activity was not detected. Therefore, mutation in PBP1 and production of beta-lactamase may not be associated with amoxicillin resistance in HP-1144. Other mechanisms must be involved in amoxicillin resistance such as decreased membrane permeability, alterations in OMPs and efflux pump.

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จุฬาลงกรณ์มหาวิทยาลัย

ABBREVIATIONS

A	adenosine
AC	amoxicillin
Ala (A)	alanine
Asn (N)	asparagine
Asp (D)	aspartic acid
Arg (R)	arginine
bp	base pair
C	cytidine
CO_2	carbon dioxide
CH	clarithromycin
^{o}C	degree Celsius
Cys (C)	cysteine
dATP	deoxyadenosine 5’-triphosphate
dCTP	deoxycytidine 5’-triphosphate
ddATP	dideoxyadenosine 5’-triphosphate
ddCTP	dideoxycytidine 5’-triphosphate
ddGTP	dideoxyguanosine 5’-triphosphate
ddTTP	dideoxytymidine 5’-triphosphate
DDW	double distilled water
ddNTPs	dideonucleotide-tri-phosphate
dGTP	deoxyguanosine 5’-triphosphate
DNA	deoxynucleic acid
dNTPs	deoxynucleotide-tri-phosphate
dTTP	deoxytymidine 5’-triphosphate
DW	distilled water
EDTA	ethylenediamine tetraacetic acid
et al.	*et alii*
E- test	epsilometer test

สถาบันวิทยบริการ
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ABBREVIATION (CONTINUED)

g	gram
G	guanosine
Gly (G)	glycine
Glu (E)	glutamic acid
Gln (Q)	glutamine
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
hr	hour
His (H)	histidine
i.e.	id test
Ile (I)	isoleucine
Lys (K)	lysine
Leu (L)	leucine
M	molar
mg	milligram
$MgCl_2$	magnesium chloride
MIC	minimum inhibitory concentration
min	minute (s)
ml	milliliter
mM	millimolar
mmol	millimole
Met (M)	methioine
NaCl	sodium chloride
Na_2HPO_4	sodium phosphate dibasic, anhydrous
NaOH	sodium hydroxide

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ABBREVIATION (CONTINUED)

NCCLS	National Committee for Clinical Laboratory Standards
Phe (F)	phenylalaine
PCR	polymerase chain reaction
Pfu	*Pyrococcus furiosus*
pmol	picomol
Pro (P)	proline
sec	second
Ser (S)	serine
T	thymidine
TAE	tris-acetate-EDTA
Thr (T)	threonine
Tris	Tris-(hydroxymethyl)-aminoethane
Trp (W)	tryptophan
Tyr (Y)	tyrosine
U	unit
µg	microgram
µl	microliter
µM	micromolar
UV	ultraviolet
V	volt

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CHAPTER I

INTRODUCTION

Helicobacter pylori is a Gram – negative curved rod or spiral microaerophilic bacterium that colonizes in the gastric mucosa of the distal portion of the human stomach (1, 2). *H. pylori* infection has a worldwide distribution. More than half of the world's population is infected. However, its prevalence varies widely according to different geographic areas, age, race and socioeconomic status. Colonization of the human stomach by *H. pylori* is usually associated with peptic ulcer disease, gastritis, gastric cancer and mucosa-associated lymphoid tissue lymphomas (2-5). Use of a single antibiotic or an antibiotic combined with bismuth is ineffective. Successful teatment of *H. pylori* infection most often use two or more antibiotics and the addition of either bismuth or a proton pump inhibitor (6-9). Triple therapy, in with a bismuth or proton pump inhibitor is combined with two antibiotics from clarithromycin, metronidazole, amoxicillin and tretracycline, is the most widely used regimen. Eradication rates of approximately 90% are obtained by using these regimens (9). However, antibiotic resistance has been reported and is one of the major causes of treatment failure. The prevalence of antibiotic resistance varies with geographical regions. Many studies have indicated that prevalence of metronidazole and clarithromycin resistance varies between 9%-80% and 1.7% - 46.4% respectively, with the highest prevalence occurring in people from developing countries (10-20). Amoxicillin resistance has been reported to be rare, however, recent data from Bangladesh, Thailand, Mexico and China have shown an increasing rate of amoxicillin resistance, ranging from 6.6% to 71.9% (10, 20-22).

Amoxicillin is a beta-lactam antibiotic that binds to penicillin-binding protiens (PBPs). Resistance to beta-lactam in Gram-negative bacteria is most often due to the production of beta-lactamase. Other resistance mechanisms include alterations in PBPs, decreased membrane permeability of antibiotic into the bacterial cell, or combination of these resistance strategies (23, 24). It has been demonstrated that resistance to amoxicillin in *H. pylori* was associated with mutations in the *pbp1* gene (25-27) and beta-lactamase activity was not detected in amoxicillin-resistant isolates

(25-31). Amoxicillin is offten used in *H. pylori* treatment regimens and there is an increasing rate of amoxicillin resistance in many countries. Therefore, it is important to update amoxicillin susceptibility in *H. pylori* isolated from Thai patients. The purpose of this study is to investigate the prevalence of amoxicillin resistance in *H. pylori* isolated from Thai patients and to determine the molecular mechanism of amoxicillin resistance in *H. pylori* by detecting mutatoins in the *pbp1* gene.

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CHAPTER II

OBJECTIVES

I. To investigate the prevalence of amoxicillin resistance in *H. pylori* isolated from Thai patients.

II. To determine mutations in the *pbp1* gene in amoxicillin-resistant *H. pylori*.

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CHAPTER III

LITERATURE REVIEW

1. BACTERIOLOGY

Helicobacter pylori was first isolated from a stomach biopsy by Warren and Marshall in 1982 (1) and initially named *Campylobacter pyloridis* because of phenotypic characteristic similar to those of *Campylobacter*. However, it was found to be different from other *Campylobacter species* and has been reclassified to a new genus, *Helicobacte*r, and given the species name *H. pylori*. *H. pylori* is a Gram-negative spiral or curved rod bacterium. It is typically 0.2 to 1.2 μm in diameter and 1.5 to 10 μm in length and have rounded ends (2). It is typically located in the mucosa of the distal portion of the human stomach. This bacterium is unusual in having sheated flagella with terminal knobs. It has four or seven unipola-sheated flagella (2). *H. pylori* has a spiral shape in young cultures but can assume coccoid orms in older cultures. It has corkscrew motility. This bacterium is microaerophilic and slow growing in culture media. Growth of *H. pylori* requires complex medium with supplementation such as whole blood, heme or serum. Colonies are small and translucent and normally take 3-5 days at 37 ^{o}C under microaerophilic condition to appear (32, 33). *H. pylori* grows best in freshly prepared medium. An important characteristic is its ability to produce an abundance of urease. Urease production is a consistent finding in *H. pylori* that colonizes the human stomach. Furthermore, *H. pylori* produces other enzymes, including catalase, oxidase, lipase, mucinase, and hemolysin. A vacuolating cytotoxin (VacA) is responsible for epithelial cell damage (34).

2. EPIDEMIOLOGY

H. pylori is found primarily in human and may be the most common bacterial infection in human. Many people appear to be infected with *H. pylori* and have no clinical disease. The outcome of infection is affected by many factors, including strain, host genotype characteristics and organism density within the stomach.

H. pylori infection has a worldwide distribution. More than half of the world's population is infected. However, the prevalence of *H. pylori* infection varies widely according to different geographic areas, age, race, and socioeconomic status (35-37). In developing countries with poor socioeconomic condition, 70% to 90% of the population is infected. In the other hand, in developed countries, the prevalence of infection is lower, ranging from 25% to 50%. In Thailand, the prevalence of *H. pylori* infection has been reported to be upto 50% in children at the age of 8 to 9 years, whereas the seroprevalence in person older than 30 years is 75% (38, 39). It appears to be no substantial reservoir of *H. pylori* aside from the human stomach. The major question of transmission is how *H. pylori* travel from the stomach of one person to that of another. Possible routes of transmission include, fecal-oral (40, 41), oral-oral and gastro-oral (42, 43).

3. CLINICAL DISEASE

H. pylori colonizes the stomach of human. This ability may be due in part to the production of the urease, which splits urea into ammonia and creates an alkaline microenvironment around the microorganism. *H. pylori* appears to specifically adhere to stomach mucosa (1), and the ammonia and possible toxins it produces may cause injury to the adjacent gastric epithelium. This leads to inflammation and is associated with clinical manifestations such as pain, gas, painful digestion, foul breath, vomiting and nausea. Tissue damage leads to ulcer in the stomach and duodenum. On the other hand, many persons harbor *H. pylori* in their stomachs and remain asymptomatic. Most persons who are infected with *H. pylori* never suffer from any symptoms related to the infection; however *H. pylori* causes chronic active and chronic persistent gastritis in adults and children. Infection with *H. pylori* also causes duodenal and gastric ulcers. Infected persons have a 2-to-6 fold increased risk of developing gastric cancer and mucosal-associated-lymphoid-type (MALT) lymphoma compared with their uninfected counterparts. The role of *H. pylori* in non-ulcer dyspepsia remains unclear (1, 44, 45).

4. DIAGNOSIS OF *H. PYLORI* INFECTION

The numerous tests and strategies have been developed to detect *H. pylori.* Infection of the stomach with *H. pylori* can be diagnosed by both invasive and noninvasive techniques (Table 1). The invasive methods require endoscopy and biopsy. These consist of histological examination, culture, urease test and polymerase chain reaction. Noninvasive methods do not require endoscopy and biopsy. These include serologic test, urea breath test and detection of *H. pylori* antigen in stool specimen. The choice of test used for diagnosis of *H. pylori* infection will depend in most cases, on the clinical information sought and the local availability and cost of individual test.

4.1 Invasive methods

4.1.1 Histology

The histological identification of *H. pylori* infection is now a widely used mean of diagnosis. Different techniques of staining (Giemsa, Warthin Starry, Hematoxylin-Eosin) are available for detection of *H. pylori*. Biopsy samples are obtained from the grossly normal appearing gastric mucosa. Prepyloric antrum has been the preferred site of biopsy. The sensitivity and specificity of histologic analysis approach 95 % to 100 % (46).

4.1.2 Urease test

A rapid urease test is an initial test of choice for the diagnosis of *H. pylori* at endoscopy in many units. It is the most rapid way to diagnose *H. pylori*. Urease activity can be measured directly in the gastric biopsy or after the microorganism has been isolated. Its low cost, ease, and excellent specificity make the rapid urease test a valuable diagnostic tool. The activity of the *H. pylori* urease enzyme splits the urea test reagent (or media agar) to form ammonia. Ammonia increases pH, which is detected by the indicator phenol red. There are numerous types of Rapid Urease Tests (RUT) on the market such as gel tests (CLOtest®) or paper tests (PyloriTek®,). In pretreatment, the sensitivity of RUTs ranges from 80 % to 95 %, while the specificity

range is 95 % to 100 % (47, 48). The sensitivity is dependent on the number of bacteria present in the gastric biopsy.

4.1.3 Polymerase Chain Reaction (PCR)

This technique is reputed to be highly sensitive and specific. The PCR consists of amplifying DNA sequences specific for *H. pylori* in gastric biopsy specimens. Factors affecting test accuracy include the choice of primers and target DNA, specimen preparation, bacterial density and technical issues regarding the PCR procedure. PCR test for *H. pylori* have been described for a number of genomic targets, such as urease gene, and virulence genes (such as *cagA, vacA, iceA*), or mutations causing macrolide resistance (49, 50).

4.1.4 Culture of *H. pylori*

Culturing *H. pylori* is difficult, timeconsuming, and expensive, and is an impractical mean of establishing the diagnosis of infection. Culture is used in controlled clinical trials of treatment of the infection. Currently, the emergence of resistant strains has rendered culture necessary (2), because it is the only method, which allows antimicrobial susceptibility testing for the isolates. To succeed in culturing *H. pylori*, special care must be taken during handling and processing of the gastric biopsy specimen (2). *H. pylori* loose its viability quickly when exposed to the environment, and biopsies should be cultured immediately to maximize the recovery of bacteria. *H. pylori* is a fastidious bacteria (2). Gastric biopsy can be transported in saline if it is to be processed within 3 hours, but transport medium must be used if the transport time will be longer. The clinical specimen should also be kept on ice before inoculation. Although, the sensitivity of culture in experienced laboratories is greater than 95 %. Culture of gastric biopsy specimens typically provides the greatest yield of *H. pylori*. Several transport media have been suggested, including normal saline, 20% glucose, and Stuart transport medium. Saline is a simple acceptable short term transport medium (51). If the culture is to be delayed more complex media such as supplemented brain heart infusion broth, horse serum, should be used (52). The transport media containing glycerol are suitable for long-term storage of biopsy specimen at -70^{o}C, or the specimens can be immediately frozen at -70^{o}C without a

fluid medium (53). Over the years, there have been a number of reports on the media that can be used for the successful culture of *H. pylori*. Originally, Marshall *et al*., (1) used brain heart infusion chocolate agar supplemented with 7 % horse blood. Subsequently, a variety of selective media and nonselective media, or a combination of both have been proposed for use in the primary isolation of *H. pylori* (1, 54, 55), but the optimal method of recovery still remains to be established. Numerous agar media including brain heart infusion agar, brucella agar, Columbia agar, Wilkins Chalgren agar, or Shirrow's agar supplemented with horse blood, horse serum, or sheep blood have been used to cultivate *H. pylori*. Primary isolation of *H. pylori* from gastric biopsy specimens on enriched media with and without antibiotic must be freshly prepared and incubated at 37 ^{o}C for 7 to 10 days under microaerophilic conditions (5-10% CO_2, 5-10% O_2, 80-90% N_2). Colonies of *H. pylori* are small, translucent and smooth. Identification involves microscopic examination and detection of enzyme (urease, catalase, and oxidase). Isolates were identified as *H. pylori* on the basis of positive urease, catalase and oxidase reactions.

4.2 Noninvasive Methods

4.2.1 Serological test

Infection with *H. pylori* stimulates a humoral immune reaction that persists as the result of continuous exposure to the bacteria. Because the antibody titers persist for many years, the test cannot be used to discriminate between past and current infection. In response to *H. pylori* infection, the immune system typically mounts a response through production of immunoglobulins to *H. pylori*-specific antigens (56). These antibodies can be detected in serum or whole – blood. Serologic test offers a fast, easy and relatively inexpensive mean of identifying patients who have been infected with *H. pylori* (56, 57). However, this method is not a useful means for confirming eradication of *H. pylori*. Serological test can only diagnose the infection and not any specific condition such as peptic ulcer or gastric cancer. Serological test was the first noninvasive method based on the detection of a specific anti- *H. pylori* IgG antibody in patient's serum (58). Persistent antibodies will lead to false- positive results. Numerous formats have been developed for the detection of specific

antibodies to *H. pylori*. These consist of latex agglutination, capillary, and enzyme-linked immunosorbent assay (ELISAs) (59).

4.2.2 Urea breath test (UBT)

H. pylori is a strong produce of urease, an enzyme that splits urea into ammonia and CO_2. The UBT is based on this principle. Carbon-labeled urea is given to a patient. The label used can be a ^{13}C or a radioactive ^{14}C (60). If *H. pylori* is present, its urease activity hydrolyses the labeled urea and the isotope is expelled as $^{13}CO_2$ or $^{14}CO_2$. If *H. pylori* is not present the labeled urea passes through the stomach intact and $^{13}CO_2$ or $^{14}CO^2$ will not be found in breath samples. ^{13}C-labeled ureas has the adventage of being non radioactive and thus safer for children and pregnant females. At certain intervals after ingestion breath sample are collected and the amount of radio-labeled CO_2 is measured. Sensitivity of UBT is in the range of 90%, whereas specificity is approximated to 100% (61).

4.2.3 Stool antigen testing

Stool antigen testing is a relatively new methodology that uses an enzyme immunoassay to detect the presence of *H. pylori* antigen in stool specimen (62, 63). It is cost effective and reliable means of diagnosis of active infection and confirming cure, and has a sensitivity and specificity comparable to those of other noninvasive tests (64). The *Helicobacter pylori* stool antigen (HpSA) test may be useful for the primary diagnosis of *H. pylori* infection. Its specificity is similar to other standard tests but its sensitivity is lower. It was found that HpSA was positive in 66.7 % of gastric ulcer disease and 80 % of duodenal ulcer disease (63).

Table 1 Summary of methods for detection of *H. pylori* (48, 60, 65, 66).

Method	Sensitivity (%)	Specificity (%)	Advantages	Disadvantages
Invasive				
Histology	93-99	95-99	Widely available; detection best with special stains; can evaluate underlying mucosal damage; gold standard	Expensive ; at least two biopsies required ; observer error; recent antibiotics or proton pump inhibitor use can lead to false negative results
Culture of biopsy specimen	77-92	100	*In vitro* antibiotic susceptibility can be determined	Expensive; organism requires special transfer and culture technique; requires up to 1 wk for results; recent antibiotics or proton pump inhibitor use can lead to false-negative results
Rapid urease test CLOtest hpFast PylorTek	89-98	93-98	Rapid results ; easy to perform ; less expensive than other invasive techniques	Formalin, simethicone, local anesthetic spray, recent antibiotics, bismuth, or proton pump inhibitor use can lead to false-negative results; poor technique or handling will affect results
Noninvasive				
Urea breath test ^{13}C ^{14}C	90-100	89-100	Inexpensive ; represents entire mucosa (not subject to biopsy sampling bias)	Antibiotics or proton pump inhibitor use can lead to false-negative results; presence of ulcer disease not determined; can be difficult to collect in children younger than 2 year of age
Serology (ELISA) HM-CAP Pylori.STAT Rapid serology FlexSure QuixVue	44-99	89-95	Inexpensive ; good for screening or epidemiologic studies in older children but not for diagnosis	Possible cross-reactivity with similar bacteria; remains positive for a variable period after successful treatment; not accurate in children younger than 6 yr of age; currently available rapid office-based tests require serum
Stool antigen testing (HpSA)	85-94	97.7	Inexpensive, more accurate test of choice in children	Stool must be collected

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5. TREATMENT OF *H. PYLORI* INFECTION

H. pylori is the cause of active chronic gastritis, a necessary factor in the peptic ulcer and a risk factor in the development of gastric cancer (67, 68). Treatment of *H. pylori* infection decreases these diseases. Maastricht guidelines (69) strongly recommended eradication therapy for unequivocally diagnose *H. pylori* positive patients with duodenal or gastric ulcer, low grade MALT gastric lymphoma, gastritis with severe early gastric cancer. Several eradication regimens have been applied, varying in the choice of antibiotic agents, dose and duration of treatment.

The most common antibiotics used for the treatment of *H. pylori* infections are clarithromycin, metronidazole, tetracycline and amoxicillin. Other antibiotics, such as fluoroquinolones or furazolidone have been used by some authors only. However, an antibiotic alone is not able to eradicate *H. pylori*. Some anti-ulcer compounds must be used, such as bismuth salts (subcitrate or subsalicylate), H_2 antogonists (ranitidine, ebrotidine, etc), proton pump inhibitors (omeprazole, lansoprazole, pantoprazole), or more recently, ranitidine bismuth citrate. These compounds play a role by increasing the pH allowing anitibiotics to act against *H. pylori* (70-73).

Monotherapy with antibiotics is unsuccessful in the treatment of *H. pylori* because of the low efficacy of currently available drugs and the rapid emergency of bacterial resistance to them. In recent years, triple therapies is first line therapy for the eradication of *H. pylori* infection (6, 9, 74-79). Triple therapy, including proton pump inhibitors (PPI), ranitidine bismuth citrate, plus 2 antibiotics (clarithromycin plus amoxicillin, tetracycline or metronidazole).

Clarithromycin

Clarithromycin is a macrolide antibiotic that inhibits bacterial protein synthesis. Its antibacterial spectrum is similar to that of erythromycin, but it is more acid-stable, better absorbed, and more effective against *H. pylori***.** As with metronidazole, resistance can develop when clarithromycin is given alone (80). Clarithromycin commonly causes an alteration of taste sensation that can be

sufficiently unpleasant to cause the cessation of treatment. It is also the most expensive of the antimicrobial drugs used to treat *H. pylori* infection.

Tetracycline

H. pylori is also very sensitive to tetracycline which inhibits bacterial protein synthesis and, like amoxicillin, appears to act luminally or topically. It is active at low pH. It should not be given to pregnant women or children because it causes permanent staining of developing teeth.

Metronidazole

Metronidazole is a mainstay of triple therapy for *H. pylori* infection. The organism ordinarily is highly sensitive to metronidazole. Metronidazole is actively secreted into the gastric juice and saliva, and therefore is active after absorption, with a half-life of 8 to 12 hours. Its activity is relatively independent of pH (81). Mutant organisms with defective nitroreductase activity are resistant to metronidazole (82), and rates of resistance are very high in areas of the world where it is used frequently for other reasons such as gynecological infections and parasitic infections (83). The side effects of metronidazole include neuropathy and, when alcohol is consumed, flushing and gastrointestinal symptoms. Tinidazole is a related drug with similar properties and side effects.

Amoxicillin

H. pylori is very sensitive *in vitro* and *in vivo* to amoxicillin. It acts by inhibiting the synthesis of bacterial cell walls. It has topical or intraluminal activity, and although stable in an acid environment, it is most active at a neutral pH (84). Unlike ampicillin, a related compound, amoxicillin is actively secreted from blood into the gastric juice,(81) and intravenous amoxicillin can therefore eradicate *H. pylori* infection (85). Bacterial resistance to amoxicillin has been rarely reported. The side effects of amoxicillin include diarrhea, allergic reactions, and pseudomembranous colitis.

6. ANTIBIOTIC RESISTANCE OF *H. PYLORI*

H. pylori is susceptible to most antibiotics *in vitro*, but when used alone, eradication of the organism from the gastric mucosa is not obtained. Factors implicated in treatment failure, include a lack of penetration by pH, lack of compliance by the patients, lack of correlation between *in vitro* susceptibility test and *in vivo* efficacy, and the presence of *H. pylori* strains with primary or acquired resistance to the antibiotics used.

In recent years, standard therapy regimens for treating *H. pylori* in fection are multiple antibiotics in combination with proton pump inhibitors (PPI). Antibiotics that are frequently included in triple therapy (6, 9, 74, 76, 86, 87) regimens are clarithromycin, metronidazole, tetracycline and amoxicillin. In addition to the lack of compliance of the patient with the treatment, emergence of antibiotic resistance has become an increasing problem leading to the therapy failure. The prevalence of anitibiotic–resistant *H. pylori* varies among different geographical areas but generally has been increasing worldwide (Table 2).

EPIDEMIOLOGY OF ANTIBIOTIC RESISTANCE IN *H. PYLORI*

Reports on the isolations of *H. pylori* with antibiotic resistance are increasing worldwide, with a high frequency in particular geographical area. The most common acquired resistance of *H. py*lori is to: metronidazole, clarithromycin, tetracycline and amoxicillin (11, 13-17, 22, 88). Prevalence of *H. pylori* resistant to antibiotic previously reported and shown in Table 2. Resistance to tetracycline is very low (11, 17, 18, 89, 90), or even absent, in most countries, such as in Spain (0 %) (90), the UK (0.5%) (89), and North Wales (0.27%) (11). However, a higher rate of tetracycline resistance was also found in Brazil (7 %) (91), Bangladesh (15 %) (10), and China (58.8 %) (21). Resistance increases with the use of antibiotic due to selective pressure. Resistance to amoxicillin is rare. Very high resistance rates to amoxicillin have been reported in some studies with a range 18 % to 71.9 % (20, 21, 91). Metronidazole resistance is more prevalent in developing countries (10) as it is widely prescribed to patients with giardiasis, amoebiasis and protozoan infections, and in women probably as a consequence of metronidazole use for gynecological conditions (92).

Metronidazole resistance varies from 21.12 % to 80 %. (10, 11, 14, 16-22, 93-95). The prevalence of primary clarithromycin resistance is increasing world wide. Prevalence rates vary from 1.7 % to 46.4 % (10-20, 22, 89-91, 94-100). A strict policy of antibiotic use is necessary to limit the spread of antibiotic resistance and surveillance programmes should be implemented at regional levels.

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Table 2 Prevalence of antibiotic resistance amongst *H. pylori* in different geographic areas.

Country/region	Year (collected)	No.of test isolates	Percentage of antibiotic resistance				Reference
			CH	MZ	TC	AC	
Netherlands	1997-1998	231	1.7	21.2	0	0	(18)
Bulgaria	1989-2000	115	12.4	15.8	ND	0	(15)
North Wales	2000-2003	363	7	24	0.27	0	(11)
Belgium	2000-2001	555	16	18	ND	0	(96)
France	1994-1999	150	21	43	ND	0	(97)
Poland	1998-2000	98	23.5	ND	ND	ND	(98)
UK	1994-1999	1064	4.4	40.3	0.5	0	(89)
Germany	1998*	172	2	21	ND	0	(99)
Spain	1991-1995	282	3.5	19.9	0	0	(90)
European multicentre	1997-1998	1,274	9.9	33.1	ND	0.8	(14)
Brazil	2000*	90	7	42	7	29	(91)
Austria	1997-2000	117	20.3	16	ND	0	(100)
Mexico	1995-1997	195	24	80	ND	18	(20)
Lebanon	2002*	45	4	29.5	2	0	(19)
Israel	2000-2001	138	46.4	60.7	0	0.72	(17)
U.S.A	1993-1999	3624	10.1 (360/3571)	36.9 (1063/2883)	ND	1.4 (48/3486)	(16)
Bangladesh	1999-2001	120	10	77.5	15	6.6	(10)
Singapore	1993-1996	495	ND	62.7	ND	ND	(93)
Korea	1996-2000	224	5.4	41.9	ND	ND	(94)
Korea	1994-1999	456	5.9	40.6	5.3	0	(95)
Japan	1996-2003	149	34.7	12.5	ND	0	(12)
Japan	1995-2000	593	11	9	ND	0.3	(13)
Thailand	2001-2002	79	19	30.4	5.1	13.9	(22)
China	1998-1999	153	ND	77.8	58.8	71.9	(21)

CH, clarithromycin; MZ, metronidazole; TC, tetracycline; AC, amoxicillin; and ND, not determined; * year published

7. AMOXICILLIN RESISTANCE

BETA-LACTAM ANTIBIOTIC

Beta-lactam belongs to a family of antibiotic, which is characterized by a beta-lactam ring. Penicillins, cephalosporins, monobactams and carbapenems are members of this antibiotic family (Figure 1). They are potent, broad-spectrum bactericidal agents of low toxicity with widespread clinical use. All beta-lactam antibiotics are bactericidal, and act by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls. The peptidoglycan layer is important for cell wall structural integrity, especially in Gram-positive organisms (101). The final transpeptidation step in the synthesis of the peptidoglycan is facilitated by transpeptidase known as penicillin-binding proteins (PBPs) (102). Beta-lactam antibiotics are analogues of D-alanyl-D-alanine the terminal amino acid residues on the precursor NAM/NAG-peptide subunits of the nascent peptidoglycan layer. The structural similarity between beta-lactam antibiotics and D-alanyl-D-alanine facilitates their binding to the active site of PBPs (103). The beta-lactam nucleus of the molecule irreversibly binds to (acylates) the serine residue of the PBP active site (103). This irreversible inhibition of the PBPs prevents the final cross-linking (transpeptidation) of the nascent peptidoglycan layer, disrupting cell wall synthesis. Inhibition of PBPs may also lead to the activation of autolytic enzymes in the bacterial cell (104).

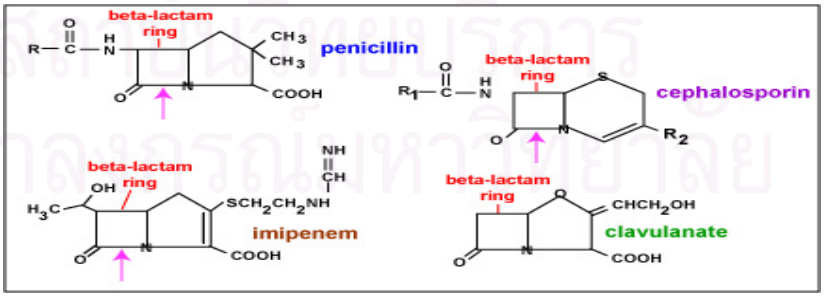

Figure 1 Chemical structure of beta-lactam antibiotics.

AMOXICILLIN

Amoxicillin is a penicillin derivative belonging to a group of beta–lactam antibiotics. This chemical modification of penicillin extends the clinical properties of antibiotic, resulting in broader spectrum activity agents Gram–negative bacteria, more complete gastrointestinal absorption and little or no effect on the interaction with food (105). The drug acts as a bactericidal agent and binds to beta-lactam receptor proteins, which are involved in cell-wall synthesis and destruction (104). Amoxicillin is often used in *H. pylori* eradication.

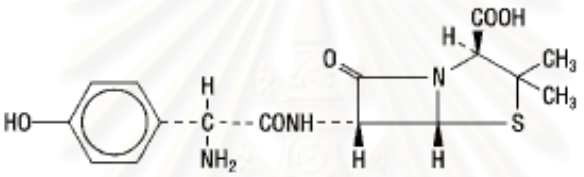

Figure 2 Chemical structure of amoxicillin.

MECHANISM OF BETA-LACTAM RESISTANCE

Resistance to beta-lactam antibiotics by Gram-negative bacteria is most commonly due to the production of beta-lactamase, either chromosomally-encoded or, more often, plasmid-mediated (106). Other important mechanisms of resistance include alterations in PBPs, decreased permeation of the antibiotic into the bacterial cell, or combinations of these resistance strategies (107). Active efflux pumps which excrete drugs, including multidrug efflux pumps, can also confer resistance to beta-lactams (108).

The major bacterial beta-lactam resistance mechanisms (Figure 3) belong to one of the following categories:

1. Enzymatic inactivation (Beta-lactamase)

Many bacteria become resistant to antibiotics by producing various bata-lactamases that are able to inactivate some forms of these drugs. Beta-lactamases break the beta-lactam ring of the antibiotics, thus destroying the drugs (24).

2. Alteration of the target site

PBPs are a group of enzymes (peptidoglycan transpeptidases) which are target sites of beta-lactam antibiotics. Changing the structure of PBPs greaty reduces affinity for binding of bacteria to these antibiotics (24).

3. Reduction in permeability

Antibiotic efflux pumps are a common way for bacteria to resist the action of numerous classes of antibiotics. Most of these pumps use protons as the motive force for efflux. A similar mechanism of resistance involves the alteration of the porin binding proteins in the Gram-negative outer membrane to limit the access of drugs to the periplasmic space (24).

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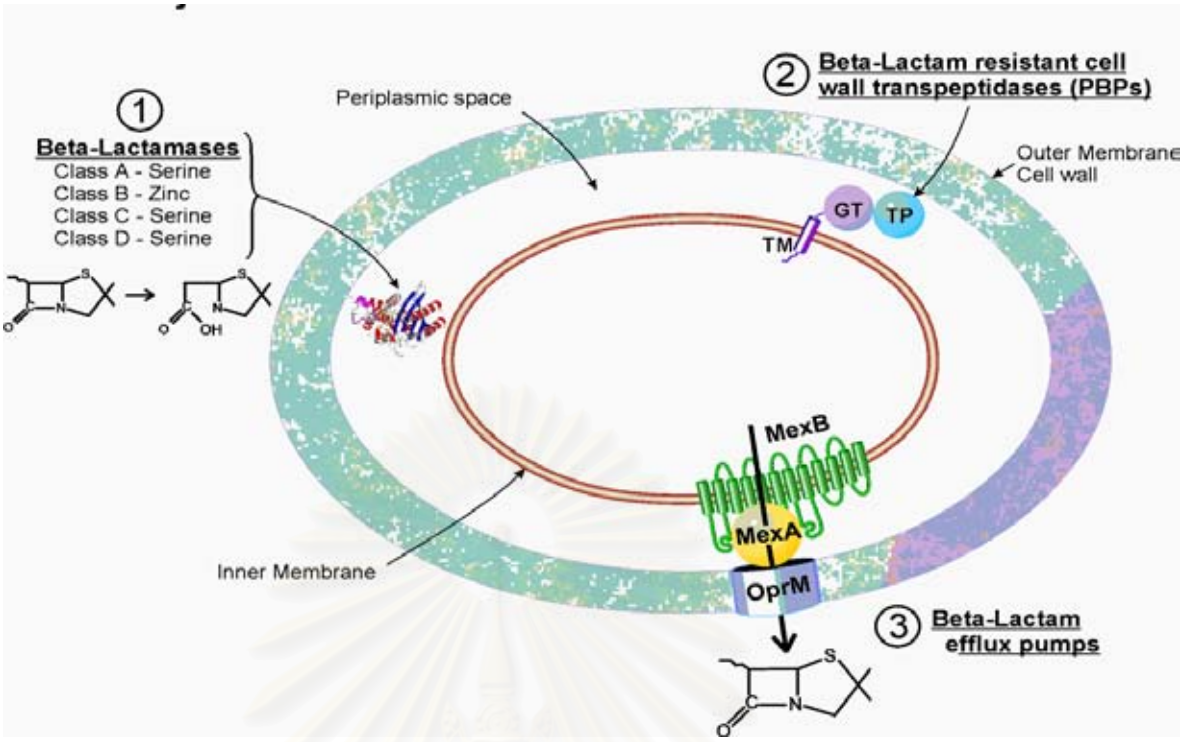

Figure 3 The major bacterial beta-lactam resistance mechanisms.

AMOXICILLIN RESISTANCE IN *H. PYLORI*

Amoxicillin is the only beta-lactam used to treat *H. pylori* infection. The molecular mechanisms of *H. pylori* strain resistance to amoxicillin are unclear. Resistance to beta-lactams in Gram-negative bacteria is most often, due to the production of beta-lactamase. However, beta-lactamase activity was not detected in *H. pylori* isolates resistant to amoxicillin (25-27, 29, 30), suggesting that this is not the mechanism in *H. pylori*. Another mechanism of bacterial resistance to amoxicillin is modification of the bacterial cell wall target, i.e., PBPs.

PBPs are a set of enzymes found in the cytoplasmic membrane of bacteria that are required for the biosynthesis of the bacterial cell wall (109, 110). PBPs catalyze the final steps of the polymerization (transglycosylation) and cross-linking (transpeptidation) of peptidoglycan , an essential component of the bacterial cell wall. PBPs are membrane–bound enzymes an targets of beta- lactam antibiotic (109). Covalent binding of beta-lactams to PBPs results in the inability of the bacterium to

built a complete cell wall, leading to cell lysis. Initial studies with *H. pylori* indicated the presence of three high-molecular-weight PBPs, designated PBP1A, PBP2 and PBP3 (29). Later a fourth, low-molecular-weight PBP, PBP4 was identified (111).

Modification of PBPs leading to decreased afftinity for antibiotics accounts for a relatively low level resistance and a stepwise increase over time in MIC values. Dore *et al*., (28) found that one of four PBPs that are normally present in amoxicillin susceptible *H. pylori* strain was missing in the amoxicillin resistance *H. pylori* strain, suggesting that modification of PBP may be responsible for the amoxicillin resistance. It has been reported that *H. pylori* resistant to amoxicillin was associated with alteration in the *pbp1* gene (25-27, 30). Kwon *et al*., (30) found that ten amino acid changes in PBP1 of amoxicillin-resistant *H. pylori* clinical isolate (MIC 64 μg/ml). These amino acids include position 406 from glutamic acid to alanine, 417 from serine to threonine, 515 from methionine to isoleucine, 535 from aspartic acid to asparagine, 543 from serine to arginine, 556 from threonine to serine, 562 from asparagine to tyrosine, 648 from lysine to glutamine, 649 from arginine to lysine and 656 from arginine to proline. In addition, Okamoto *et al*., (27) reported four amino acid changes in PBP1 of amoxicillin-resistant *H.pylori* clinical isolate (MIC 8 ug/m/), including at the position 69 from alanine to valine, 556 from threonine to serine, 562 from asparagine to tyrosine, 593 from threonine to alanine and an insertion of amino acid at the position 464 as glutamic acid. Paul *et al*., (25) demonstrated that mutations in PBP1 at the position 414 from serine to arginine, 484 from tyrosine to cysteine, 541 from threonine to isoleucine and 600 from proline to threonine(25)of amoxicillin-resistant strain from laboratory mutant. Gerrits *et al*., (26) suggested that alteration in PBP1 in amoxicillin-resistant isolate at the position 414 from serine to arginine conferred resistance.

CHAPTER IV

MATERIALS AND METHODS

The chemical agents used in this study were molecular biology grade. Name list of all media, chemical reagents materials, instruments and reagents were shown in Appendix I.

1. BACTERIAL STRAINS

Helicobactor pylori were isolated from Gastric biopsies from 357 patients who underwent upper gastrointestinal endoscopy at King Chulalongkorn Memorial Hospital between August 2003 and June 2004. Four gastric biopsy specimens were obtained from the antrum of each patient and placed in transport medium (1ml of 0.85 % sterile normal saline). All specimens were transported to the laboratory on ice and processed within 2 hours.

2. ISOLATION OF *H. PYLORI* ISOLATES

Two pieces of gastric biopsies from each patient were ground and cultured on selective and non-selective media modified from Tee *et al*., (112). Non-selective medium is Columbia blood agar containing 7% sheep blood and 7% horse serum. Selective medium is Columbia blood agar with antibiotic supplement containing vancomycin (10 mg/L), cefsulodin (5 mg/L), trimethoprim (5 mg/L), and amphotericin B (5 mg/L). Plates were incubated at 37 ^{o} C under microaerophilic conditions (10 % CO_2, 5 % O_2 and 85 % $N_{2)}$ produced by a gas generating system (CampyGen, Oxoid, England) in anaerobic jar for 5-10 days.

3. IDENTIFICATION OF *H. PYLORI* ISOLATES

Colonies were identified as *H. pylori* by typical morphology on Gram-stained smear and positive urease, catalase and oxidase test. *H. pylori* NCTC 11637 was used as a positive control.

4. BIOCHEMICAL TESTS

4.1 Catalase test

The enzyme catalase breaks down hydrogen peroxide into oxygen. This results in the visible formation of bubbles of oxygen. Catalase activity is detected by putting a loop full of colonies into a drop of 3% (V/V) hydrogen peroxide on a glass slide. If the microorganism is catalase-positive, bubbles will appear due to the liberation of oxygen.

4.2 Oxidase test

The oxidase test detects the presence of cytochrome oxidase. This enzyme can oxidize the substrate N, N,Ń,Ń-tetramethyl-*p*-phenylenediamine dihydrochloride (Sigma, U.S.A) and to produce a purple color. To test for oxidase, transfer colonies with a loop onto filter paper soaked with oxidase reagent. A dark purple color will appear within 10 seconds if the microorganism has an enzyme, cytochrome oxidase.

4.3 Urease test

The purpose of the urease test is to detect whether an microorganism possesses the enzyme urease which hydrolyzes urea. The urea agar contains phenol red indicator, which turns pink at an alkaline pH. When urea is hydrolyzed, it releases ammonia, which causes an alkaline reaction and a vivid pink color develops. Urease activity is detected by inoculating a loop full of the organism onto slope of the urea agar slant. A positive pink color occurs in a few minutes.

5. PRESERVATION OF *H. PYLORI* ISOLATES

H. pylori isolates were subcultured on Columbia blood agar with 7% sheep blood and 7% horse serum (GibcoBRL, U.S.A) at 37 ^{o}C under microaerophilic conditions for 3 days and were transferred by loop into 1 ml Brain heart infusion broth (Oxoid, England) containing 20% glycerol (V/V) (18). The strains were kept at-70 ^{o}C until used.

6. ANTIMICROBIAL SUSCEPTIBILITY TEST

All *H. pylori* isolates were tested for amoxicillin susceptibility. The minimal inhibitory concentration (MIC) of amoxicillin was examined by the epsilometer test (Etest, AB Biodish, Solan, Sweden) and an agar dilution. The MIC is the lowest concentration of antimicrobial agents required to inhibit the growth of a microorganism *in vitro*.

6.1 Epsilometer test (E-test)

The E-test is a plastic strip containing a predefined, continuous and exponential antibiotic gradient on one side and graded continuous MIC scale (0.016 to 256 μg/ml). Columbia blood agars with a depth of 4 mm in plastic plate (90 mm) were used to determine the MIC by E-test. The media and E-test amoxicillin strips must be allowed to reach room temperature prior to use. The inoculum was prepared by the direct colony suspension method. *H. pylori* strains were grown on Columbia blood agar, at 37^{o}C under microaerophilic conditions for 3 days. Subsequently, the selected colonies were transferred into brain heart infusion broth (Oxoid, USA). Suspension of *H. pylori* were adjusted turbidity to a McFarland standard no 3 (9×10^{8} CFU/ml) and 1 ml of the suspensions were added onto Columbia blood agar (17). The suspensions were spreaded by bent rod, and the excess suspensions were removed. The inoculated agar plates were allowed to air dry for 15 minutes at room temperature prior to apply amoxicillin E-test strips (AB Biodisk, Solna, Sweden). E-test strips were placed on the agar surface by sterile forceps. After an incubation period of 3 days at 37^{o}C under microaerophilic conditions produced by a

gasgenerating system (CampyGen Pack, Oxoid). The MIC was defined by the point of complete inhibition of all growth, including hazes and isolated colonies. Amoxicillin resistance was reported when the MIC value was greater than 0.5 μg/ml (12-15, 113, 114).

6.2 Agar dilution

H. pylori strains which were resistant to amoxicillin were confirmed by the agar dilution. Amoxicillin resistance in *H. pylori* was determined when the MIC value was greater than 0.5 μg/ml. Stock solutions of amoxicillin (Sigma Chemical Co., St Louis, MO, USA) were prepared at 10 mg/ml. Agar dilution plates were prepared using Columbia blood agar containing 7% (V/V) sheep blood, 7% (V/V) horse serum supplemented with twofold serial dilutions of amoxicillin. Fresh *H. pylori* isolates (2 to 3 days cultures) were prepared in Brain heart infusion broth (Oxoid, USA.) and adjusted to a no.3 McFarland standard (9×10^8 CFU / ml) (17). Mark the plates so that the orientation is obvious. Transfer diluted bacterial suspensions to the wells of an inoculum replicating apparatus. Use the apparatus to transfer the inocula to the series of agar plates, including a control plate without antimicrobial agent. Allow the inoculum spots to dry at room temperature before inverting the plates for incubation. The plates were incubated at 37 ^{o}C under microaerophilic conditions for 3 days. The MIC was investigated as the lowest amoxicillin concentration preventing visible growth.

7. DETEMINATION OF BETA-LACTAMASE PRODUCTION

The production of beta-lactamase by *H. pylori* strains was tested by the chromogenic cephalosporin method (115). Several colonies of *H. pylori* were selected and smear over the paper imprenated with nitrocefin solution of 500 μg/ml (Oxoid, England), a chromogenic cephalosporin. The presence of beta-lactamase was indicated by a change in the color of filter paper disc from yellow to pink. Activity was determined absent if no color change had occurred after 2 hours of incubation at room temperature. *Neisseria gonorrhoeae* beta-lactamase positive (Laboratory strain) was used as positive control.

8. AMPLIFICATION OF *pbp 1* GENE

8.1 DNA Extraction

The extraction of *H. pylori* DNA was performed by QIAamp DNA Mini kit (QIAGEN, Germany). The DNA was purified according to the manufacturer's directions. *H. pylori* colonies were scraped from the culture plate with inoculation loop and suspended with 180 µl of ALT. Twenty microliters of proteinase K were added, mixed by vortexing, and incubated at 56 ^{0}C until bacterial cell were completely lysed. The samples were then added by 200 µl of buffer AL. After well mixing the suspensions were heated at 70 ^{0}C for 10 min, and briefly centrifuged to remove drops from inside the lid. The samples were added by 200 µl of ethanol and mixed by pulse-vortexing for 15 sec. The supernatants were then transferred into spin columns and centrifuged at 8,000 rpm for 1 min. Place the QIAamp spin columns in a clean 2 ml collection tube and discard the tube containing the filtrate. QIAamp spin columns were carefully opened and 500 µl of buffer AW1 were added and centrifuged at 8,000 rpm for 1 min. The filtrates were then discarded. QIAamp spin columns were washed with 500 µl of AW2 and centrifuged at 14,000 rpm for 3 minutes. QIAamp spin columns were placed in a clean 1.5 microcentrifuge tube and added with 200 µl of buffer AE. The samples were incubated at room temperature for 1 minute and centrifuged at 8,000 rpm for 1 minute. Extracted DNA were stored at –20 ^{0}C.

8.2 DNA amplification by polymerase chain reaction (PCR)

8.2.1 Primer

Primers for amplification of *pbp1* were designed with primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The sequence data appear in GenBank under accession no. AE 000573. A fragment of 2,200 bp was amplified by using PBP1-F1 and PBP1-R1 primers (Table 3).

8.2.2 Amplification of the *pbp1* gene by PCR

Primer PBP1-F1 and PBP1-R1 were used for amplification of the entire *pbp1* gene in a 50 μl PCR reaction mixture. All PCR reagents were purchased from Furmentas, USA. The PCR mixture was as followed; 10X PCR buffer (20mM Tris-HCl (pH 8.8), 10 mMKCl, 10mM $(NH_4)_2$ $SO_{4)}$, 1.5mMgCl2, 200 μMdNTP mixture (dNTPs; dATP, dCTP, dGTP, dTTP), 20 pmol of each primer, 2 U *Pfu* DNA polymerase, and 5 μl of DNA template. The reactions were run for 30 cycles through a temperature profile of 94 ^{o}C 1 minute (denaturation), 55 ^{o}C for 90 seconds (annealing), 72 ^{o}C 2 minutes (extension) and final extention at 72 ^{o}C for 10 minutes.

8.3 Analysis of PCR product

The PCR products were analyzed on 1 % agarose gels (Pronalisa, Spain) in TBE buffer containing 0.5 μg/ml ethidium bromide (Sigma, USA) was prepared. Ten miroliters of PCR products were mixed with 3 μl of gel loading buffer (20 % ficoll, 0.05 % bromophenol blue). The electrophoresis was carried out at 80 volts for 50 minutes. The amplified products were visualized on of UV light transilluminator. A 1 Kb DNA ladder was used as a DNA size marker.

9. DETECTION OF POINT MUTATIONS IN *pbp 1* GENE BY DNA SEQUENCING

9.1 Purification of PCR product

The PCR products of 2,200 bp were purified by QIAquick PCR Purification Kit as described by the manufacturer (QIAGEN, GmbH, Germany). The QIAquick system is combination of spin-column technology with the selective birding properties of a uniquely- designed silica-gel membrane. DNA was absorbed to the silica- membrane in the presence of high salt while contaminants pass through the column. The PCR products were added 5 volumes of buffer PB (Contains quanidine hydrochloride and isopropanol) to 1 volume of PCR sample. After well mixing, the PCR sample were then transferred into QIAquick spin column in a provided 2 ml

collection tube and centrifuged at 13,000 rpm for 30-60 sec. Discard flow – through and place the QIAquick column back into the same tube. Collection tubes were re-used to reduce plastic waste. QIAquick spin column was carefully opened and added 750 μl of buffer PE (washing buffer) and centrifuged at 13,000 rpm for 30-60 sec. QIAquick spin columns were placed in a clean 1.5 microcentrifuge tube and added with 50 μl of buffer EB (10 mMTris-Cl, pH 8.5) The samples were incubated at room temperature for 1 minute and centrifuged at 13,000 rpm for 30-60 sec. The purified PCR product were stored at -20 ^{0}C. The concentration of DNA was determined by spectrophotometer (BIO RAD, U.S.A).

9.2 Primers for DNA sequencing

Primers for DNA sequenced of *pbp1* were designed with primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) base on the sequence data in GenBank under accession no. AE 000573. A fragment of 2,200 bp was amplified using PBP1-F1 and PBP1-R1 primers. The primers used for sequencing are shown in Table 3.

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Table 3 Sequences of the oligonucleotides used as primers for DNA sequencing the *pbp1* gene of *H. pylori* (GenBank under accession number AE000573).

Primers	Sequence (5' to 3')	Locations
PBP1-F1	CCT ACG GTT TCT AAA CCC CTT T	6766-6745
PBP1-F2	GCG CGC TAT GAT TAA AAA CG	6335-6316
PBP1-F3	ACG GGT TAA AAA CTC AAG GCTA	5817-5796
PBP1-F4	AAC CTT GCA AGA AGC CTT GA	5369-5350
PBP1-F5	TTG GTT CAT TGG CTT TAC CC	4946-4927
PBP1-R1	ATC AAG CGG TGA GTA TCC TTG T	4567-4588
PBP1-R2	GAG CCA AAC TCC CTG TGC	5005-5022
PBP1-R3	GCG CGG TAT CAG GGA TTT	5461-5478
PBP1-R4	GCT CGT TAG AAG AAA TCC AGC CTA	5923-5946
PBP1-R5	CGT GTG AGC ACC ATG TTT TT	6243-6262

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9.3 Preparation of sequencing reactions

PCR products were sequenced using primers shown in Table 3 with ABI prime Big Dye terminator cycle sequencing ready reaction kit (PE Applied Biosystem, Foster, Ca). The sequence reaction required 4 μl of Big Dye® Therminator as demonstrated by manufacturer, with 3.2 pmole of primer and 50 ng of purified PCR product template in a total volume of 10 μl. The sequencing cycle was examined using 25 cycles of denaturation at 96 ^{0}C for 30 sec, annealing at 50 ^{0}C for 10 sec and extension at 60 ^{0}C for 4 min and hold at 4 ^{0}C until ready to purify. The PCR product was purified with ethanol / EDTA precipitation before capillary electrophoresis was run for sequencing analysis.

9.4 Analysis

The nucleotide sequence and the deduced protein sequence were analyzed with the software available over the Internet at the National Center for Biotechnology Information (http: // www.ncbi.nlm.nih.gov/BLAST),
Multalin (www.toulouse.inra.fr/multalin.html) and ExPASy (www.expasy.org/).

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CHAPTER V

RESULTS

1. BACTERIAL STRAINS

Gastric biopsy samples were obtained from 357 patients who underwent upper gastrointestinal endoscopy at King Chulalongkorn Memorial Hospital between August 2003 and June 2004. There were 134 men and 223 women with mean age 48 years (range, 16-93 years). Of all patients, 289 had non-ulcer dyspepsia, 56 had gastric ulcer, and 12 had duodenal ulcer (Table 4).

Fifty *Helicobacter pylori* isolates were obtained from 357 patients (14 %, 50/357). All clinical isolates included 33 strains (11.4% , 33/289) from non-ulcer dyspepsia patients , mean age 49 years, range 26-79 years, male / female : 9/24, 14 strains (25% , 14/56) from gastric ulcer patients, mean age 57 years, range 35-89 years, male / female : 9/5, and 3 strains (25%, 3/12) from duodenal ulcer patients, mean age 53 years, range 38-73 years, male / female : 3 / 0 (Table 4, Appendix IV: Table 7).

A total of fifty *H. pylori* isolates were identified as *H. pylori* based on morphological and biochemical tests. Colonies grown on Columbia blood agar were examined for morphology and biochemical characteristics. Colonies of *H. pylori* are small translucent to gray (Figure 4). Gram stain of *H. pylori* colonies shows a range of shapes from short rods to curved rod and occasionally S-shaped spirals (Figure 5). Isolates were identified as *H. pylori* on the basis of positive urease, catalase and oxidase reactions (Figure 6-8).

Table 4 *H. pylori* isolates and patient groups.

Patient group*	Fifty *H. pylori* isolates		
	No.of *H. pylori* isolates (%)	Age	Gender
		Mean/Range	Male/Female
NUD (n=289)	33 (11.4)	49/26-79	9/24
GU (n=56)	14 (25)	57/35-89	9/5
DU (n=12)	3 (25)	53/38-73	3/0

*****NUD, non-ulcer dyspepsia; GU, gastric ulcer; DU, duodenal ulcer

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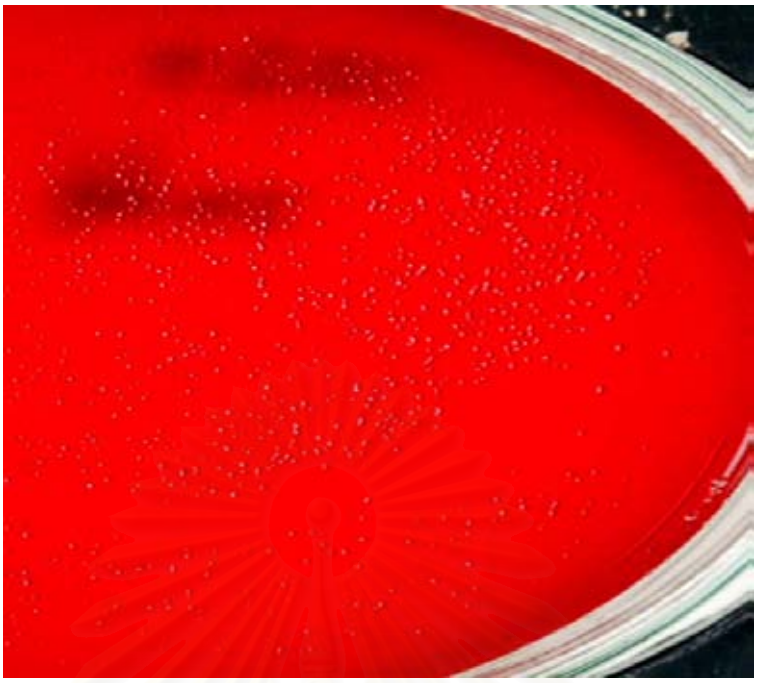

Figure 4 Colonies of *H. pylori* on Columbia blood agar plate.

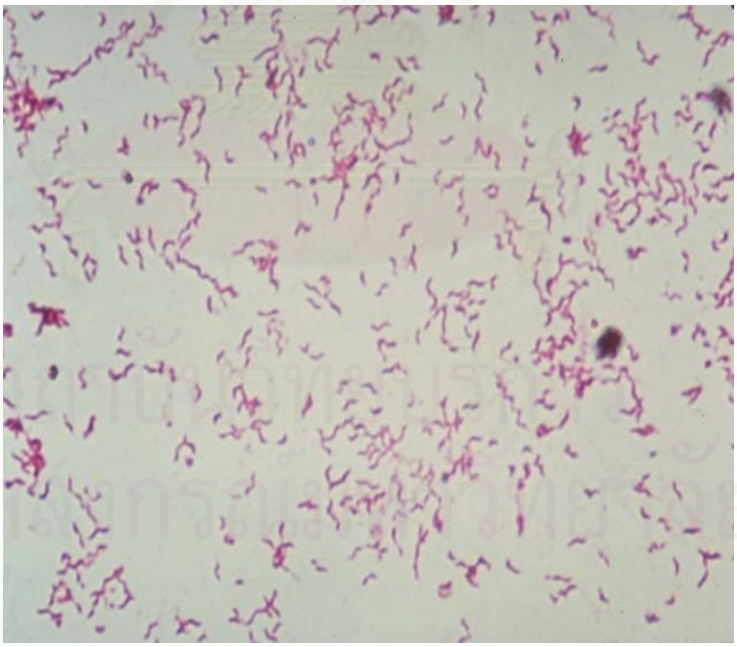

Figure 5 Gram stain of *H. pylori*.

Figure 6

Urease test: *H. pylori* is positive for urease test

Figure 7 Oxidase test:

***H. pylori* is positive for oxidase test**

Figure 8 Catalase test: *H. pylori* is positive for catalase test

2. DETERMINATION OF THE AMOXICILLIN SUSCEPTIBILITY OF *H. PYLORI*

The MIC results for the 50 *H. pylori* isolates are shown in Table 7 (Appendix IV) and Figure 9. For MICs determined by the E-test, strains were considered resistant when the MIC was > 0.5 μg/ml for amoxicillin (13-15, 113, 114). Distribution of MICs of amoxicillin for the 50 *H. pylori* isolates are shown in Figure 9. It was demonstrated that MICs of *H. pylori* isolates ranged from < 0.016 to 0.75 μg/ml. MIC required to inhibit 50 % (MIC_{50}) and 90% (MIC_{90}) of isolates are <0.016 μg/ml and 0.016 μg/ml, respectively.

Of the 50 isolates, 49 (98%) were susceptible to amoxicillin (MICs range = < 0.016-0.25 μg/ml) and only one isolate was resistant (MIC = 0.75 μg/ml). Of these isolates, 70 % (35/50) had the MIC of < 0.016 μg/ml, 24% (12/50) had the MIC of 0.016 μg/ml, and two isolates had the MIC of 0.047 and 0.25 μg/ml respectively. The remaining one isolate (2 %), HP-1144, was resistant to amoxicillin (MIC 0.75 μg/ml). The MIC of amoxicillin-resistant *H. pylori* was confirmed by the agar dilution method. There was a slightly higher MIC of HP-1144 (MIC=1μg/ml) by agar dilution comparing by E-test.

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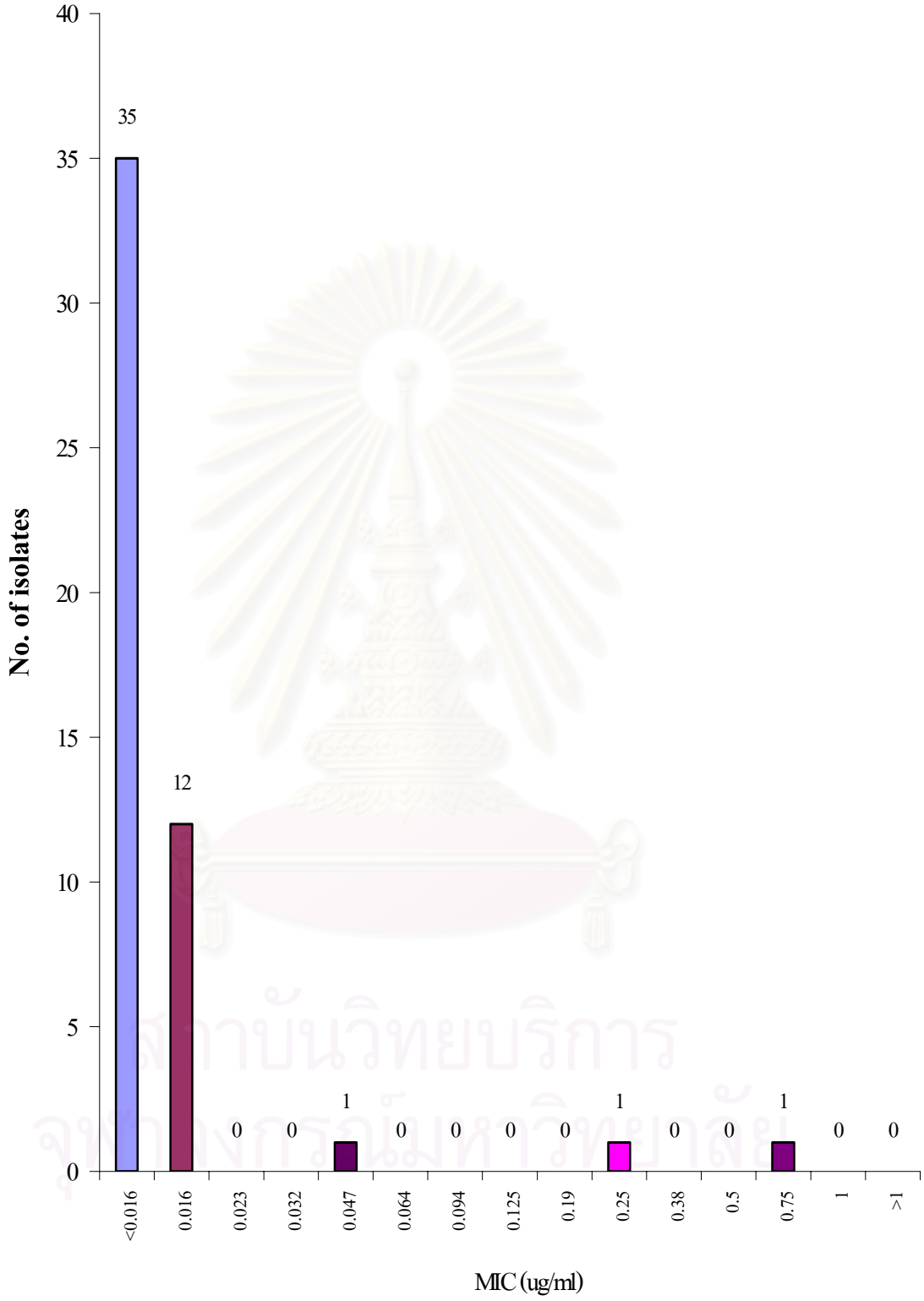

Figure 9 Distribution of amoxicillin MICs determined by the E-test.

3. DETECTION OF BETA-LACTAMASE

In this study, only one *H. pylori* isolate was resistant to amoxicillin (MIC 0.75 μg/ml). It was tested for production of beta–lactamase using the chromogenic cephalosporin method. However, the activity of beta – lactamase was not found in amoxicillin–resistant *H. pylori* strain, HP-1144.

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4. AMPLIFICATION OF *pbp1* GENE

Six *H. pylori* isolates were screened for the presence of *pbp1* gene by PCR with primers PBP-F1 and PBP-R1. Of the 6 isolates, 5 were susceptible to amoxicillin (MICs range = < 0.016 – 0.25 μg/ml) and one isolate, HP-1144, was resistant (MIC = 0.75μg/ml). These susceptible strains included *H. pylori* with different MIC; *H. pylori* NCTC 11637 (MIC = 0.016 μg/ml), HP-1174 (MIC= <0.016 μg/ml), HP-1163 (MIC= 0.016 μg/ml), HP-870 (MIC= 0.047 μg/ml), HP-1028 (MIC= 0.25 μg/ml) and HP-1144 (MIC= 0.75 μg/ml). PCR products of 2200 bp were present in all strains, including *H. pylori* NCTC 11637 (lane 3), amoxicillin- susceptible *H. pylori* strains (lane 4-7) and amoxicillin – resistant *H. pylori* strain (lane 8) as shown in Figure 10.

Figure 10 Agarose gel electrophoresis of PCR products of *pbp1* gene (2200 bp) from *H. pylori* isolates.

Lane 1: 1 Kb DNA marker Lane 2: Double distilled water

Lane 3: *H. pylori* NCTC 11637 (MIC =0.016 μg/ml)

Lane 4: HP–1174 (MIC < 0.016 μg/ml)

Lane 5: HP–1163 (MIC= 0.016 μg/ml)

Lane 6: HP–870 (MIC =0.047 μg/ml)

Lane 7: HP–1028 (MIC =0.25 μg/ml)

Lane 8: HP–1144 (MIC =0.75 μg/ml)

5. DETECTION OF POINT MUTATION IN *pbp1* GENE BY DNA SEQUENCING

Nucleotide sequences of *pbp1* gene from the 6 *H. pylori* isolates including a resistant isolate (HP-1144, MIC 0.75 μg/ml) and susceptible isolates (*H. pylori* NCTC 11637, HP-1174, HP-1163, HP-870 and HP-1028) were determined by sequencing and amino acid changes in PBP1 are shown in Table 5 and Appendix IV (Figure 11-12). The comparison of the PBP1 sequences of all six stains with *H. pylori* 26695 from Genbank was investigated. The results demonstrated that there was diversity of amino acid substitutions in PBP1 (Table 5) both amoxicillin–susceptible and amoxicillin - resistant *H. pylori* isolates. Alignments of the PBP1 from amoxicillin – resistant, amoxicillin-susceptible isolates and *H. pylori* 26695 did not highlight any clustering of amino acid substitutions in PBP1 of amoxicillin resistant isolate (Table 5).

Comparison of the PBP1 sequences of all six strains revealed amino acid substitutions. There were 10-18 amino acid substitutions in PBP1 among all isolates sequenced. One amino acid deletion at position 400 and forty-two amino acid substitutions were found in both amoxicillin- susceptible and amoxicillin-resistant isolates. Of the 42 amino acid substitutions, 27 were found in sensitive strains (positions 13, 16, 45, 114, 120, 242, 243, 322, 332, 352, 374, 392, 406, 408, 414, 432, 469, 534, 547, 556, 589, 593, 595, 611, 653, 654 and 656) and 15 shared by both amoxicillin-resistant and sensitive *H. pylori* isolates (positions 17, 35, 79, 125, 148, 324, 479, 504, 508, 509, 515, 535, 543, 648 and 649) (Table 5). However, the variability of nucleotide substitutions and amino acid substitutions sequence in the PBP1 did not relate to the amoxicillin MICs, suggesting that these amino acid changes did not directly relate to the functional activity of PBP1. Amino acid substitutions in PBP1 in amoxicillin-sensitive isolates suggested there is genetic diversity of *pbp1* gene of *H. pylori*.

It has been demonstrated that transpeptidase domain of PBP1 of *H. pylori* 26695 has a catalytic center consisting of motif 1 (SXXK at 368 – 371), motif 2 (SLN at 433 – 435) and motif 3 (KTG at 555 – 557) when aligned with the sequence of *E. coil* PBP1a(102). In this study, the resistant isolate, HP-1144, had no amino acid substitution in these 3 motifs but one amoxicillin-susceptible isolate, HP-1028, with

the MIC of 0.25 μg/ml had mutation in the KTG motif changing from KTG to KSG (Table 6).

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Table 5 Amino acid changes in PBP1 in amoxicillin-susceptible and amoxicillin-resistant *H. pylori* isolates.

Strains	MICs μg/ml	Amino acid at position :																					
		13	16	17	35	45	79	114	120	125	148	242	243	322	324	332	352	374	392	400	406	408	414
Susceptible strains																							
26695*	0.016	F	V	M	I	V	I	Y	T	F	I	G	A	N	D	M	K	V	T	N	E	N	S
HP-1174	<0.016	.	.	I	T	.	V	T	.	L	L	.	.	.	Y	.	.	.	.	.	.	.	.
HP-1163	0.016	.	I	I	.	I	.	.	.	L	L	.	.	D	.	.	.	.	A	.	V	S	.
HPC	0.016	.	I	I	.	I	.	.	.	L	L	.	.	D	.	.	.	.	.	.	.	.	.
HP-870	0.047	V	I	V	.	.	.	.	.	L	.	S	S	.	.	.	.	.	.	.	.	.	.
HP-1028	0.25	.	I	.	.	.	.	.	A	L	.	.	.	.	.	I	E	L	.	**	.	.	R
Resistant strain																							
HP-1144	0.75	.	.	I	T	.	V	.	.	L	L	.	.	.	Y	.	.	.	.	.	.	.	.

*; GenBank accession no.AE000573, HPC; *H. pylori* NCTC 11637, **; deletion of amino acid
. ; Same as for 26695

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Table 5 Amino acid changes in PBP1 in amoxicillin-susceptible and amoxicillin-resistant *H. pylori* isolates (Continued).

Strains	MICs μg/ml	Amino acid at position :																					
		432	469	479	504	508	509	515	534	535	543	547	556	589	593	595	611	648	649	653	654	656
Susceptible strains																						
26695*	0.016	H	V	D	N	D	V	M	M	D	S	I	T	S	T	G	A	K	R	S	E	R
HP-1174	<0.016	.	.	E	D	N	I	I	.	N	N	.	.	.	.	.	.	Q	K	.	.	.
HP-1163	0.016	L	.	.	.	.	.	.	I	N	R	T	.	.	.	.	.	.	.	.	.	.
HPC	0.016	.	.	.	.	.	.	.	I	N	R	T	.	.	.	.	.	.	.	.	.	.
HP-870	0.047	.	G	E	D	.	.	.	.	N	.	.	.	G	A	S	.	Q	K	G	K	P
HP-1028	0.25	.	.	E	D	.	.	I	.	N	.	.	S	G	.	.	S	.	.	.	.	P
Resistant strain																						
HP-1144	0.75	.	.	E	D	N	I	I	.	N	N	.	.	.	.	.	.	Q	K	.	.	.

*; GenBank accession no.AE000573, HPC; *H. pylori* NCTC 11637, **; deletion of amino acid

• **;** Same as for 26695

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Table 6 Sequences of the three conserved amino acid motifs of PBP1.

Strains	**Change in amino acids of conserved PBP1 sites making up active penicillin-binding site of PBP.**		
	Motif 1 Position 368-371	**Motif 2 Position 433-435**	**Motif 3 Position 555-557**
HP-26695	SAIK	SLN	KTG
HPC	SAIK	SLN	KTG
HP-1163	SAIK	SLN	KTG
HP-1174	SAIK	SLN	KTG
HP-870	SAIK	SLN	KTG
HP-1028	SAIK	SLN	**KSG**
HP-1144	SAIK	SLN	KTG

HPC; *H. pylori* NCTC 11637

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CHAPTER VI

DISCUSSION

Helicobacter pylori infection is responsible for most cases of peptic ulcer disease and successful treatment of the infection results in cure of the disease. Triple therapy, in with a bismuth or proton pump inhibitor is combined with two antibiotics from clarithromycin, metronidazole, amoxicillin and tetracycline, is the most widely used regimen. Eradication rates of approximately 90 % are obtained by using these regimens (9). However, antibiotic resistance has been reported and is one of the major causes of treatment failure.

The prevalence of amoxicillin resistance varies with geographical regions. Amoxicillin resistance has been reported to be rare, however, recent data have shown an increasing rate of amoxicillin resistance in Bangladesh (6.6%) (10), Thailand (13.9%) (22), Mexico (18%) (20) and China (71.9%) (21). This study shows low rate of *H. pylori* resistant to amoxicillin (2%). The prevalence of amoxicillin resistance rate was higher in previous studies from Brazil (29%) (91), Mexico (18%) (20), Bangladesh (6.6%) (10) and China (71.9%) (21). The difference in rates of resistance is due to the geographical origin of population and the cutoff values used for amoxicillin resistance. Variability occurs between laboratories with amoxicillin – resistant breakpoints described from as low as 0.5 μg/ml to as high as 8 μg/ml (11, 13-15, 19, 113, 116, 117). Similar results have been reported in the studies from Korea (0%) (95), Philippines (0%) (118) Japan (0.3%) (13) , USA (1.4%) (16), Israel (0.72%) (17) and European multicentre (0.8%) (14). However, the present study, amoxicillin resistance rate (2%) in *H. pylori* isolate from Thai patients was lower than in the study by Tangmankongworakoon *et al*.,(13.9%) (22). The difference may be partly from the different group of patients and testing parameters (medium, size of inoculums, incubation time). We had only 50 *H. pylori* isolates, therefore, more isolates are needed to clearly demonstrated the prevalence of amoxicillin resistance.

In this study, MIC_{50} and MIC_{90} of amoxicillin showed that amoxicillin was potent against *H. pylori* as the MIC_{90} was 0.016 μg/ml and only one low-level resistant isolate was found (MIC 0.75 μg/ml). This suggests that amoxicillin is

potential alternative agent for the treatment of clarithromycin-resistant and metronidazole-resistant *H. pylori* in Thai patients and further clinical studies are need to confirm these data *in vivo*.

Alterations in PBPs resulting in reduced binding ability can lead to resistance to beta-lactam. Previous studies demonstrated that resistance to amoxicillin in *H. pylori* was associated with mutation in the *pbp1* gene (25-27, 30). Similar findinges were observed in *Streptococcus pneumoniae* (119-121), *S. mitis* (122) and *Neisseria gonorrhoeae* (123). Seventeen distinct PBP1 mutations have been reported to be associated with amoxicillin resistance in *H. pylori*, i.e., at the position 69 from alanine to valine, 406 from glutamic acid to alanine, 414 from serine to arginine, 417 from serine to threonine, 484 from tyrosine to cysteine, 515 from methionine to isoleucine, 535 from aspartic acid to asparagine, 541 from threonine to isoleucine, 543 from serine to arginine, 556 from threonine to serine, 562 from asparagine to tyrosine, 593 from threonine to alanine, 600 from proline to threonine, 648 from lysine to glutamine, 649 from arginine to lysine and 656 from arginine to proline and one amino acid insertion at position 464 (25-27, 30). However, the results from these studies obtained from only 1-2 *H. pylori* isolates (MICs range= 8 μg/ml to 64 μg/ml).

When compared with amino acid sequence of PBP1 of *H. pylori* 26695 from GenBank, the result from this study showed forty-two different amino acid substitutions in both amoxicillin-sensitive and resistant isolates at positions 13, 16, 17, 35, 45, 79, 114, 120, 125, 148, 242, 243, 322, 324, 332, 352, 374, 392, 406, 408, 414, 432, 469, 479, 504, 508, 509, 515, 534, 535, 543, 547, 556, 589, 593, 595, 611, 648, 649, 653, 654 , 656 and one amino acid deletion at position 400. Fifteen amino acid changes were shared among resistant and susceptible strains. All mutations in the resistant isolate were found in susceptible isolates (positions 17, 35, 79, 125, 148, 324, 479, 504, 508, 509, 515, 535, 543, 648 and 649). The results are different from the study by Okamoto *et al.*, (27) which demonstrated five mutations in PBP1 of amoxicillin-resistant clinical isolate (MIC 8 μg/ml), including amino acid substitutions at the position 69, 556, 562, 593 and one amino acid insertion at position 464. Our study found amino acid substitutions at the position 556 and 593 in susceptible isolates. Kwon *et al.*,(30) demonstrated that high-level beta-lactam

resistance in clinical *H. pylori* isolate (MIC 64 μg/ml) was associated with alterations in PBP1, resulting in the following amino acid substitutions at the positions 406, 417, 515, 535, 543, 556, 562, 648, 649 and 656. Our study demonstrated that amino acid substitutions in PBP1 at the positions 406, 515, 535, 543, 556, 648, 649 and 656 were also found in amoxicillin-susceptible isolates. Gerrit *et al.* (26) reported that amino acid substitution at the position 414 in PBP1 was responsible for amoxicillin resistance. In contrast, our study found that amino acid substitution at position 414 was present in amoxicillin-susceptible isolate (HP-1028). Amino acid substitutions at position 414, 484, 541 and 600 in an amoxicillin-resistant strain were also reported by Paul *et al.* (25) However, none of these mutations was found in amoxicillin-resistant isolate, HP-1144.

The transpeptidase domain of PBP1 of *H. pylori* 26695 has a catalytic centre consisting of motif 1 (SXXK at 368-371), motif 2 (SLN at 433-435) and motif 3 (KTG at 555-557) when aligned with the sequence of *E. coli* PBP1a (102). It has been reported that the amoxicillin-resistant isolates had a KSG sequence in motif 3 instead of the KTG sequence conserved in other strains (27, 30). In contrast, we did not find mutations in these motifs in amoxicillin-resistant isolate but mutation in KTG motif was occurred in amoxicillin-susceptible strain. The susceptible strain HP-1028 (MIC = 0.25μg/ml) has a KSG sequence in motif 3 instead of KTG sequence conserved in other strains. Therefore, alteration in KTG motif may not the sole contributor to the mechanism of amoxicillin resistance. Other mechanisms of resistance must be involved such as decreased permeation of the antibiotic in to the bacterial cell, alterations in OMPs and active efflux pumps.

HP-1028 with the MIC of 0.25 μg/ml, had mutations in KTG motif and amino acid substitution at position 414. Although these mutations were not found in our resistant strain (HP-1144), it may be associated with increased amoxicillin MIC as this isolate had the MIC 15.6 times more than the MIC of most strains (MIC_{90} = 0.016 μg/ml).

It has been demonstrated that beta-lactam resistance in *H. pylori* was not mediated by beta-lactamase (25-30). Similar to other studies, beta-lactamase activity was not detected in amoxicillin-resistant isolate (HP-1144).

The present study showed that mutations in the *pbp1* gene may not be associated with low-level amoxicillin resistance in HP-1144, an amoxicillin-resistant clinical isolate, as amino acid substitutions found in this isolate were also present in sensitive strains. Other mechanisms of resistance must play a role in this amoxicillin-resistant isolate, including decreased permeation of the antibiotic into the bacterial cell, alterations in OMPs and active efflux pumps.

When compared the PBP1 sequences of HP-1144 and HP-1174 (MIC <0.016 μg/ml), the results showed that all except one amino acid at position 114 were identical. Therefore, amino acid substitution at position 114 may be involved in the mechanism of amoxicillin resistance. For further study, site-directed mutagenesis should be done to investigate the role of mutation at position 114.

The limitation of this study is that we had only one amoxicillin-resistant isolate with low-level amoxicillin resitance. Much more amoxicillin-resistant clinical *H. pylori* isolates are required to gain more results on the PBP1 mutations associated with amoxicillin resistance. Interestingly, our results showed that mutations in PBP1 at positions 406, 414, 515, 535, 543, 556, 593, 648, 649 and 656 which others investigators reported to be linked to amoxicillin resistance were present in our amoxicillin susceptible isolates. Although there were no particular amino acid substitution associated with amoxicillin resistance in this study, a number and types of amino acid changes may cause structureal changes in PBP1 leading to decreased affinity of PBP1 for amoxicillin.

CHAPTER VII

CONCLUSION

In recent years, standard therapy regimens for treating *Helicobacter pylori* infection are multiple antibiotics in combination with proton pump inhibitors (PPI). Antibiotics that are frequently included in triple therapy (6, 9, 74, 76, 86, 87) regimens are clarithromycin, metronidazole, tetracycline and amoxicillin. Amoxicillin is an important component of combination therapies for *H. pylori* eradication and there is an increasing rate of amoxicillin resistance in many countries.

Fifty *H. pylori* isolates were obtained from 357 patients (14%, 50/357) who underwent upper gastrointestinal endoscopy at King Chulalongkorn Memorial Hospital between August 2003 and June 2004. The minimum inhibitory concentration (MIC) of amoxicillin was examined by the E-test. Of the 50 isolates, 49 (98%) were susceptible to amoxicillin and only one isolate was resistant (2%) (MIC 0.75 μg/ml). MIC_{50} and MIC_{90} were <0.016 and 0.016 μg/ml, respectively. This suggests that amoxicillin is potential alternative agent for the treatment of clarithromycin-resistant and metronidazole-resistant *H. pylori* in Thai patients and further clinical studies are needed to confirm these data *in vivo*.

Mutations in the *pbp1* gene were analyzed by PCR and DNA sequencing. The DNA sequences of *pbp1* were determined by sequencing both amoxicillin – susceptible and amoxicillin – resistant *H. pylori* isolates. Of the 6 isolates, 5 were susceptible to amoxicillin (MICs range = < 0.016 – 0.25 μg/ml) and one isolate, HP-1144, was resistant to amoxicillin (MIC = 0.75μg/ml). When compared with amino acid sequence of PBP1 of *H. pylori* 26695 from GenBank, there were forty-two different amino acid substitutions in both amoxicillin-sensitive and resistant isolates and one amino acid deletion. Fifteen amino acid changes were shared among resistant and susceptible strains. All mutations in the resistant isolate, HP-1144, were found in susceptible isolates.

Interestingly, our results showed that mutations in PBP1 at positions 406, 414, 515, 535, 543, 556, 593, 648, 649 and 656 which others investigators reported to be linked to amoxicillin resistance were present in our amoxicillin susceptible isolates. Beta-lactamase activity was not detected in HP-1144.

Therefore, mutation in PBP1 and production of beta-lactamase may not be associated with amoxicillin resistance in HP-1144. Other mechanisms must be involved in amoxicillin resistance such as decreased membrane permeability, alterations in OMPs and efflux pump.

สถาบันวิทยบริการ
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REFERENCES

(1) Warren JR MB. Unidentified curve bacilli on gastric epithelium in active chronic gastritis. Lancet 1983:1273-5.

(2) Dunn BE, Cohen H, Blaser MJ. *Helicobacter pylori.* Clin Microbiol Rev 1997;10(4):720-41.

(3) Yoshimura T, Shimoyama T, Tanaka M, Sasaki Y, Fukuda S, Munakata A. Gastric mucosal inflammation and epithelial cell turnover are associated with gastric cancer in patients with *Helicobacter pylori* infection. J Clin Pathol 2000;53(7):532-6.

(4) Mueller A, O'Rourke J, Grimm J, Guillemin K, Dixon MF, Lee A, *et al*. Distinct gene expression profiles characterize the histopathological stages of disease in *Helicobacter*-induced mucosa-associated lymphoid tissue lymphoma. Proc Natl Acad Sci U S A 2003;100(3):1292-7.

(5) Moayyedi P, Axon AT, Feltbower R, Duffett S, Crocombe W, Braunholtz D, *et al.* Relation of adult lifestyle and socioeconomic factors to the prevalence of *Helicobacter pylori* infection. Int J Epidemiol 2002;31(3):624-31.

(6) Murakami K, Fujioka T, Okimoto T, Sato R, Kodama M, Nasu M. Drug combinations with amoxycillin reduce selection of clarithromycin resistance during *Helicobacter pylori* eradication therapy. Int J Antimicrob Agents 2002;19(1):67-70.

(7) Perri F, Festa V, Merla A, Quitadamo M, Clemente R, Andriulli A. Amoxicillin/tetracycline combinations are inadequate as alternative therapies for *Helicobacter pylori* infection. Helicobacter 2002;7(2):99-104.

(8) Nagahara A, Miwa H, Ogawa K, Kurosawa A, Ohkura R, Iida N, *et al*. Addition of metronidazole to rabeprazole-amoxicillin-clarithromycin regimen for *Helicobacter pylori* infection provides an excellent cure rate with five-day therapy. Helicobacter 2000;5(2):88-93.

(9) Bellelis P, Samano ES, Nunes RC, Ribeiro Lde M, Chehter EZ, Catapani WR. Efficacy of a triple therapy for *Helicobacter pylori* eradication in a well-developed urban area in Brazil. Sao Paulo Med J 2004;122(2):73-5.

(10) Nahar S, Mukhopadhyay AK, Khan R, Ahmad MM, Datta S, Chattopadhyay S, *et al*. Antimicrobial susceptibility of *Helicobacter pylori* strains isolated in Bangladesh. J Clin Microbiol 2004;42(10):4856-8.

(11) Elviss NC, Owen RJ, Xerry J, Walker AM, Davies K. *Helicobacter pylori* antibiotic resistance patterns and genotypes in adult dyspeptic patients from a regional population in North Wales. J Antimicrob Chemother 2004;54(2):435-40.

12 Kato S, Konno M, Maisawa S, Tajiri H, Yoshimura N, Shimizu T, *et al*. Results of triple eradication therapy in Japanese children: a retrospective multicenter study. J Gastroenterol 2004;39(9):838-43.

(13) Perez Aldana L, Kato M, Nakagawa S, Kawarasaki M, Nagasako T, Mizushima T, *et al*. The relationship between consumption of antimicrobial agents and the prevalence of primary *Helicobacter pylori* resistance. Helicobacter 2002;7(5):306-9.

(14) Glupczynski Y, Megraud F, Lopez-Brea M, Andersen LP. European multicentre survey of in vitro antimicrobial resistance in *Helicobacter pylori*. Eur J Clin Microbiol Infect Dis 2001;20(11):820-3.

(15) Boyanova L, Koumanova R, Gergova G, Popova M, Mitov I, Kovacheva Y, *et al*. Prevalence of resistant *Helicobacter pylori* isolates in Bulgarian children. J Med Microbiol 2002;51(9):786-90.

(16) Meyer JM, Silliman NP, Wang W, Siepman NY, Sugg JE, Morris D, *et al*. Risk factors for *Helicobacter pylori* resistance in the United States: the surveillance of *H. pylori* antimicrobial resistance partnership (SHARP) study, 1993-1999. Ann Intern Med 2002;136(1):13-24.

(17) Samra Z, Shmuely H, Niv Y, Dinari G, Passaro DJ, Geler A, *et al*. Resistance of *Helicobacter pylori* isolated in Israel to metronidazole, clarithromycin, tetracycline, amoxicillin and cefixime. J Antimicrob Chemother 2002;49(6):1023-6.

(18) Debets-Ossenkopp YJ, Herscheid AJ, Pot RG, Kuipers EJ, Kusters JG, Vandenbroucke-Grauls CM. Prevalence of *Helicobacter pylori* resistance to metronidazole, clarithromycin, amoxycillin, tetracycline and trovafloxacin in The Netherlands. J Antimicrob Chemother 1999;43(4):511-5.

(19) Sharara AI, Chedid M, Araj GF, Barada KA, Mourad FH. Prevalence of *Helicobacter pylori* resistance to metronidazole, clarithromycin, amoxycillin and tetracycline in Lebanon. Int J Antimicrob Agents 2002;19(2):155-8.

(20) Torres J, Camorlinga-Ponce M, Perez-Perez G, Madrazo-De la Garza A, Dehesa M, Gonzalez-Valencia G, *et al*. Increasing multidrug resistance in *Helicobacter pylori* strains isolated from children and adults in Mexico. J Clin Microbiol 2001;39(7):2677-80.

(21) Wu H, Shi XD, Wang HT, Liu JX. Resistance of *Helicobacter pylori* to metronidazole, tetracycline and amoxycillin. J Antimicrob Chemother 2000;46(1):121-3.

(22) Tangmankongworakoon N, Mahachai V, Thong-Ngam D, Vilaichone RK, Tumwasorn S, Kullavanijaya P. Pattern of drug resistant *Helicobacter pylori* in dyspeptic patients in Thailand. J Med Assoc Thai 2003;86 Suppl 2:S439-44.

(23) Megraud F. Basis for the management of drug-resistant *Helicobacter pylori* infection. Drugs 2004;64(17):1893-904.

(24) Sefton AM. Mechanisms of antimicrobial resistance: their clinical relevance in the new millennium. Drugs 2002;62(4):557-66.

(25) Paul R, Postius S, Melchers K, Schafer KP. Mutations of the *Helicobacter pylori* genes *rdxA* and *pbp1* cause resistance against metronidazole and amoxicillin. Antimicrob Agents Chemother 2001;45(3):962-5.

(26) Gerrits MM, Schuijffel D, van Zwet AA, Kuipers EJ, Vandenbroucke-Grauls CM, Kusters JG. Alterations in penicillin-binding protein 1A confer resistance to beta-lactam antibiotics in *Helicobacter pylori*. Antimicrob Agents Chemother 2002;46(7):2229-33.

(27) Okamoto T, Yoshiyama H, Nakazawa T, Park ID, Chang MW, Yanai H, *et al*. A change in PBP1 is involved in amoxicillin resistance of clinical isolates of *Helicobacter pylori.* J Antimicrob Chemother 2002;50(6):849-56.

(28) Dore MP, Graham DY, Sepulveda AR. Different penicillin-binding protein profiles in amoxicillin-resistant *Helicobacter pylori.* Helicobacter 1999;4(3):154-61.

(29) DeLoney CR, Schiller NL. Characterization of an In vitro-selected amoxicillin-resistant strain of *Helicobacter pylori.* Antimicrob Agents Chemother 2000;44(12):3368-73.

(30) Kwon DH, Dore MP, Kim JJ, Kato M, Lee M, Wu JY, *et al*. High-level beta-lactam resistance associated with acquired multidrug resistance in *Helicobacter pylori*. Antimicrob Agents Chemother 2003;47(7):2169-78.

(31) Dore MP, Osato MS, Realdi G, Mura I, Graham DY, Sepulveda AR. Amoxycillin tolerance in *Helicobacter pylori*. J Antimicrob Chemother 1999;43(1):47-54.

(32) Bukholm G, Tannaes T, Nedenskov P, Esbensen Y, Grav HJ, Hovig T, *et al.* Colony variation of *Helicobacter pylori*: pathogenic potential is correlated to cell wall lipid composition. Scand J Gastroenterol 1997;32(5):445-54.

(33) Fawcett PT, Gibney KM, Vinette KM. *Helicobacter pylori* can be induced to assume the morphology of *Helicobacter heilmannii*. J Clin Microbiol 1999;37(4):1045-8.

(34) Kim SY, Woo CW, Lee YM, Son BR, Kim JW, Chae HB, *et al*. Genotyping CagA, VacA subtype, IceA1, and BabA of *Helicobacter pylori* isolates from Korean patients, and their association with gastroduodenal diseases. J Korean Med Sci 2001;16(5):579-84.

(35) Murray LJ, McCrum EE, Evans AE, Bamford KB. Epidemiology of *Helicobacter pylori* infection among 4742 randomly selected subjects from Northern Ireland. Int J Epidemiol 1997;26(4):880-7.

(36) Souto FJ, Fontes CJ, Rocha GA, de Oliveira AM, Mendes EN, Queiroz DM. Prevalence of *Helicobacter pylori* infection in a rural area of the state of Mato Grosso, Brazil. Mem Inst Oswaldo Cruz 1998;93(2):171-4.

(37) Frenck RW, Jr., Clemens J. *Helicobacter* in the developing world. Microbes Infect 2003;5(8):705-13.

(38) Bodhidatta L, Hoge CW, Churnratanakul S, Nirdnoy W, Sampathanukul P, Tungtaem C, *et al.* Diagnosis of *Helicobacter pylori* infection in a developing country: comparison of two ELISAs and a seroprevalence study. J Infect Dis 1993;168(6):1549-53.

(39) Perez-Perez GI, Taylor DN, Bodhidatta L, Wongsrichanalai J, Baze WB, Dunn BE, *et al*. Seroprevalence of *Helicobacter pylori* infections in Thailand. J Infect Dis 1990;161(6):1237-41.

(40) Furuta T, Kamata T, Takashima M, Futami H, Arai H, Hanai H, *et al*. Study of transmission routes of *Helicobacter pylori* in relation to seroprevalence of hepatitis A virus. J Clin Microbiol 1997;35(7):1891-3.

(41) Megraud F. Transmission of *Helicobacter pylori*: faecal-oral versus oral-oral route. Aliment Pharmacol Ther 1995;9 Suppl 2:85-91.

(42) Leung WK, Siu KL, Kwok CK, Chan SY, Sung R, Sung JJ. Isolation of *Helicobacter pylori* from vomitus in children and its implication in gastro-oral transmission. Am J Gastroenterol 1999;94(10):2881-4.

(43) De Schryver AA, Van Winckel MA. *Helicobacter pylori* infection: epidemiology and occupational risk for health care workers. Ann Acad Med Singapore 2001;30(5):457-63.

(44) Leontiadis GI, Minopoulos GI, Maltezos E, Kotsiou S, Manolas KI, Simopoulos K, *et al*. Effects of *Helicobacter pylori* infection on gastric emptying rate in patients with non-ulcer dyspepsia. World J Gastroenterol 2004;10(12):1750-4.

(45) Zhang C, Yamada N, Wu YL, Wen M, Matsuhisa T, Matsukura N. *Helicobacter pylori* infection, glandular atrophy and intestinal metaplasia in superficial gastritis, gastric erosion, erosive gastritis, gastric ulcer and early gastric cancer. World J Gastroenterol 2005;11(6):791-6.

(46) Genta RM, Graham DY. Comparison of biopsy sites for the histopathologic diagnosis of *Helicobacter pylori*: a topographic study of *H. pylori* density and distribution. Gastrointest Endosc 1994;40(3):342-5.

(47) Laine L, Chun D, Stein C, El-Beblawi I, Sharma V, Chandrasoma P. The influence of size or number of biopsies on rapid urease test results: a prospective evaluation. Gastrointest Endosc 1996;43(1):49-53.

(48) Midolo P, Marshall BJ. Accurate diagnosis of *Helicobacter pylori*. Urease tests. Gastroenterol Clin North Am 2000;29(4):871-8.

(49) Hao Q, Li Y, Zhang ZJ, Liu Y, Gao H. New mutation points in 23S rRNA gene associated with *Helicobacter pylori* resistance to clarithromycin in northeast China. World J Gastroenterol 2004;10(7):1075-7.

(50) Schabereiter-Gurtner C, Hirschl AM, Dragosics B, Hufnagl P, Puz S, Kovach Z, *et al*. Novel real-time PCR assay for detection of *Helicobacter pylori* infection and simultaneous clarithromycin susceptibility testing of stool and biopsy specimens. J Clin Microbiol 2004;42(10):4512-8.

(51) Soltesz V, Zeeberg B, Wadstrom T. Optimal survival of *Helicobacter pylori* under various transport conditions. J Clin Microbiol 1992;30(6):1453-6.

(52) Siu LK, Leung WK, Cheng AF, Sung JY, Ling TK, Ling JM, *et al*. Evaluation of a selective transport medium for gastric biopsy specimens to be cultured for *Helicobacter pylori.* J Clin Microbiol 1998;36(10):3048-50.

(53) Han SW, Flamm R, Hachem CY, Kim HY, Clarridge JE, Evans DG, *et al*. Transport and storage of *Helicobacter pylori* from gastric mucosal biopsies and clinical isolates. Eur J Clin Microbiol Infect Dis 1995;14(4):349-52.

(54) Stevenson TH, Lucia LM, Acuff GR. Development of a selective medium for isolation of *Helicobacter pylori* from cattle and beef samples. Appl Environ Microbiol 2000;66(2):723-7.

(55) Roosendaal R, Kuipers EJ, Pena AS, de Graaff J. Recovery of *Helicobacter pylori* from gastric biopsy specimens is not dependent on the transport medium used. J Clin Microbiol 1995;33(10):2798-800.

(56) Lepper PM, Moricke A, Vogt K, Bode G, Trautmann M. Comparison of different criteria for interpretation of immunoglobulin G immunoblotting results for diagnosis of *Helicobacter pylori* infection. Clin Diagn Lab Immunol 2004;11(3):569-76.

(57) Loy CT, Irwig LM, Katelaris PH, Talley NJ. Do commercial serological kits for *Helicobacter pylori* infection differ in accuracy? A meta-analysis. Am J Gastroenterol 1996;91(6):1138-44.

(58) Hanvivatvong O, Pongpanich A, Thong-Ngam D, Thammacharoenrach N, Kullavanijaya P. Evaluation of commercial immunoassays for detection of antibody against *Helicobacter pylori* in Thai dyspeptic patients. Clin Diagn Lab Immunol 2004;11(3):618-20.

(59) Locatelli A, Catapani WR, Gomes CR, Jr., Silva CB, Waisberg J. Detection of anti-*Helicobacter pylori* antibodies in serum and duodenal fluid in peptic gastroduodenal disease. World J Gastroenterol 2004;10(20):2997-3000.

(60) Ohara S, Kato M, Saito M, Fukuda S, Kato C, Hamada S, *et al*. Comparison between a new 13C-urea breath test, using a film-coated tablet, and the conventional 13C-urea breath test for the detection of *Helicobacter pylori* infection. J Gastroenterol 2004;39(7):621-8.

(61) Graham DY, Klein PD. Accurate diagnosis of *Helicobacter pylori.* 13C-urea breath test. Gastroenterol Clin North Am 2000;29(4):885-93, x.

(62) Weingart V, Russmann H, Koletzko S, Weingart J, Hochter W, Sackmann M. Sensitivity of a novel stool antigen test for detection of *Helicobacter pylori* in adult outpatients before and after eradication therapy. J Clin Microbiol 2004;42(3):1319-21.

(63) Syam AF, Rani AA, Abdullah M, Manan C, Makmun D, Simadibrata M, *et al.* Accuracy of *Helicobacter pylori* stool antigen for the detection of Helicobacter pylori infection in dyspeptic patients. World J Gastroenterol 2005;11(3):386-8.

(64) Crone J, Symonds E, Campbell F, Butler R. Evaluation of a monoclonal antibody-based test for detection of *Helicobacter pylori*-specific antigen in stool samples from mice. Clin Diagn Lab Immunol 2004;11(4):799-800.

(65) Taj Y, Essa F, Kazmi SU, Abdullah E. Sensitivity and specificity of various diagnostic tests in the detection of *Helicobacter pylori*. J Coll Physicians Surg Pak 2003;13(2):90-3.

(66) Hachem CY, Clarridge JE, Evans DG, Graham DY. Comparison of agar based media for primary isolation of *Helicobacter pylori.* J Clin Pathol 1995;48(8):714-6.

(67) Kato S, Nishino Y, Ozawa K, Konno M, Maisawa S, Toyoda S, *et al.* The prevalence of *Helicobacter pylori* in Japanese children with gastritis or peptic ulcer disease. J Gastroenterol 2004;39(8):734-8.

(68) Kirsch C, Madisch A, Piehler P, Bayerdorffer E, Stolte M, Miehlke S. *Helicobacter pylori* in gastric corpus of patients 20 years after partial gastric resection. World J Gastroenterol 2004;10(17):2557-9.

(69) Go MF. What are the host factors that place an individual at risk for *Helicobacter pylori*-associated disease? Gastroenterology 1997;113(6 Suppl):S15-20.

(70) Alarcon T, Domingo D, Sanchez I, Diaz de Rojas F, Lopez-Brea M. In vitro activity of omeprazole in combination with several antimicrobial agents against clinical isolates of *Helicobacter pylori.* Eur J Clin Microbiol Infect Dis 1996;15(12):937-40.

(71) Lopez-Brea M, Domingo D, Sanchez I, Prieto N, Alarcon T. Study of the combination of ranitidine bismuth citrate and metronidazole against metronidazole-resistant *Helicobacter pylori* clinical isolates. J Antimicrob Chemother 1998;42(3):309-14.

(72) Midolo PD, Turnidge JD, Lambert JR. Bactericidal activity and synergy studies of proton pump inhibitors and antibiotics against *Helicobacter pylori in vitro*. J Antimicrob Chemother 1997;39(3):331-7.

(73) Meyer JM, Ryu S, Pendland SL, Danziger LH. *In vitro* synergy testing of clarithromycin and 14-hydroxyclarithromycin with amoxicillin or bismuth subsalicylate against *Helicobacter pylori*. Antimicrob Agents Chemother 1997;41(7):1607-8.

(74) Gottrand F, Kalach N, Spyckerelle C, Guimber D, Mougenot JF, Tounian P, *et al.* Omeprazole combined with amoxicillin and clarithromycin in the eradication of *Helicobacter pylori* in children with gastritis: A prospective randomized double-blind trial. J Pediatr 2001;139(5):664-8.

(75) Graham DY, Opekun AR, Belson G, El-Zimaity HM, Carlson MR. Novel bismuth-metronidazole-tetracycline triple-layer tablet for treatment of *Helicobacter pylori*. Aliment Pharmacol Ther 2005;21(2):165-8.

(76) Fock KM, Chelvam P, Lim SG. Triple therapy in the eradication of *Helicobacter pylori* in patients with duodenal ulcer disease: results of a multicentre study in South-East Asia. South-East Asia Multicenter Study Group. Aliment Pharmacol Ther 2000;14(2):225-31.

(77) Kawabata H, Habu Y, Tomioka H, Kutsumi H, Kobayashi M, Oyasu K, *et al.* Effect of different proton pump inhibitors, differences in CYP2C19 genotype and antibiotic resistance on the eradication rate of *Helicobacter pylori* infection by a 1-week regimen of proton pump inhibitor, amoxicillin and clarithromycin. Aliment Pharmacol Ther 2003;17(2):259-64.

(78) Koivisto TT, Rautelin HI, Voutilainen ME, Heikkinen MT, Koskenpato JP, Farkkila MA. First-line eradication therapy for *Helicobacter pylori* in primary health care based on antibiotic resistance: results of three eradication regimens. Aliment Pharmacol Ther 2005;21(6):773-82.

(79) Isomoto H, Furusu H, Ohnita K, Wen CY, Inoue K, Kohno S. Sofalcone, a mucoprotective agent, increases the cure rate of *Helicobacter pylori* infection when combined with rabeprazole, amoxicillin and clarithromycin. World J Gastroenterol 2005;11(11):1629-33.

(80) Peterson WL, Graham DY, Marshall B, Blaser MJ, Genta RM, Klein PD, *et al.* Clarithromycin as monotherapy for eradication of *Helicobacter pylori*: a randomized, double-blind trial. Am J Gastroenterol 1993;88(11):1860-4.

(81) van Zanten SJ, Goldie J, Hollingsworth J, Silletti C, Richardson H, Hunt RH. Secretion of intravenously administered antibiotics in gastric juice: implications for management of *Helicobacter pylori*. J Clin Pathol 1992;45(3):225-7.

(82) Cederbrant G, Kahlmeter G, Ljungh A. Proposed mechanism for metronidazole resistance in *Helicobacter pylori*. J Antimicrob Chemother 1992;29(2):115-20.

(83) Results of a multicentre European survey in 1991 of metronidazole resistance in *Helicobacter pylori*. European Study Group on Antibiotic Susceptibility of *Helicobacter pylori*. Eur J Clin Microbiol Infect Dis 1992;11(9):777-81.

(84) Grayson ML, Eliopoulos GM, Ferraro MJ, Moellering RC, Jr. Effect of varying pH on the susceptibility of *Campylobacter pylori* to antimicrobial agents. Eur J Clin Microbiol Infect Dis 1989;8(10):888-9.

(85) Adamek RJ, Wegener M, Opferkuch W, Ruhl GH. Successful *Helicobacter pylori* eradication: a systemic effect of antibiotics? Am J Gastroenterol 1993;88(5):792-3.

(86) Wong WM, Gu Q, Wang WH, Fung FM, Berg DE, Lai KC, *et al*. Effects of primary metronidazole and clarithromycin resistance to *Helicobacter pylori* on omeprazole, metronidazole, and clarithromycin triple-therapy regimen in a region with high rates of metronidazole resistance. Clin Infect Dis 2003;37(7):882-9.

(87) Bazzoli F, Pozzato P, Rokkas T. *Helicobacter pylori*: the challenge in therapy. Helicobacter 2002;7 Suppl 1:43-9.

(88) Hosking SW, Ling TK, Yung MY, Cheng A, Chung SC, Leung JW, *et al.* Randomised controlled trial of short term treatment to eradicate *Helicobacter pylori* in patients with duodenal ulcer. Bmj 1992;305(6852):502-4.

(89) Parsons HK, Carter MJ, Sanders DS, Winstanley T, Lobo AJ. *Helicobacter pylori* antimicrobial resistance in the United Kingdom: the effect of age,

sex and socio-economic status. Aliment Pharmacol Ther 2001;15(9):1473-8.

(90) Lopez-Brea M, Domingo D, Sanchez I, Alarcon T. Evolution of resistance to metronidazole and clarithromycin in *Helicobacter pylori* clinical isolates from Spain. J Antimicrob Chemother 1997;40(2):279-81.

(91) Mendonca S, Ecclissato C, Sartori MS, Godoy AP, Guerzoni RA, Degger M, *et al.* Prevalence of *Helicobacter pylori* resistance to metronidazole, clarithromycin, amoxicillin, tetracycline, and furazolidone in Brazil. Helicobacter 2000;5(2):79-83.

(92) Banatvala N, Davies GR, Abdi Y, Clements L, Rampton DS, Hardie JM, *et al.* High prevalence of *Helicobacter pylori* metronidazole resistance in migrants to east London: relation with previous nitroimidazole exposure and gastroduodenal disease. Gut 1994;35(11):1562-6.

(93) Teo EK, Fock KM, Ng TM, Khor CJ, Tan AL. Metronidazole-resistant *Helicobacter pylori* in an urban Asian population. J Gastroenterol Hepatol 2000;15(5):494-7.

(94) Eun CS, Han DS, Park JY, Jeon YC, Hahm JS, Kim KS, *et al*. Changing pattern of antimicrobial resistance of *Helicobacter pylori* in Korean patients with peptic ulcer diseases. J Gastroenterol 2003;38(5):436-41.

(95) Kim JJ, Reddy R, Lee M, Kim JG, El-Zaatari FA, Osato MS, *et al.* Analysis of metronidazole, clarithromycin and tetracycline resistance of *Helicobacter pylori* isolates from Korea. J Antimicrob Chemother 2001;47(4):459-61.

(96) Bontems P, Devaster JM, Corvaglia L, Dezsofi A, Van Den Borre C, Goutier S, *et al*. Twelve year observation of primary and secondary antibiotic-resistant *Helicobacter pylori* strains in children. Pediatr Infect Dis J 2001;20(11):1033-8.

(97) Kalach N, Bergeret M, Benhamou PH, Dupont C, Raymond J. High levels of resistance to metronidazole and clarithromycin in *Helicobacter pylori* strains in children. J Clin Microbiol 2001;39(1):394-7.

(98) Dzierzanowska-Fangrat K, Rozynek E, Jozwiak P, Celinska-Cedro D, Madalinski K, Dzierzanowska D. Primary resistance to clarithromycin in clinical strains of *Helicobacter pylori* isolated from children in Poland. Int J Antimicrob Agents 2001;18(4):387-90.

(99) Adamek RJ, Suerbaum S, Pfaffenbach B, Opferkuch W. Primary and acquired *Helicobacter pylori* resistance to clarithromycin, metronidazole, and amoxicillin--influence on treatment outcome. Am J Gastroenterol 1998;93(3):386-9.

(100) Crone J, Granditsch G, Huber WD, Binder C, Innerhofer A, Amann G, *et al.* *Helicobacter pylori* in children and adolescents: increase of primary clarithromycin resistance, 1997-2000. J Pediatr Gastroenterol Nutr 2003;36(3):368-71.

(101) Georgopapadakou NH, Liu FY. Penicillin-binding proteins in bacteria. Antimicrob Agents Chemother 1980;18(1):148-57.

(102) Goffin C, Ghuysen JM. Multimodular penicillin-binding proteins: an enigmatic family of orthologs and paralogs. Microbiol Mol Biol Rev 1998;62(4):1079-93.

(103) Bush K, Smith SA, Ohringer S, Tanaka SK, Bonner DP. Improved sensitivity in assays for binding of novel beta-lactam antibiotics to penicillin-binding proteins of *Escherichia coli.* Antimicrob Agents Chemother 1987;31(8):1271-3.

(104) Spratt BG. Properties of the penicillin-binding proteins of *Escherichia coli* K12. Eur J Biochem 1977;72(2):341-52.

(105) Vakil N, Cutler A. Ten-day triple therapy with ranitidine bismuth citrate, amoxicillin, and clarithromycin in eradicating *Helicobacter pylori.* Am J Gastroenterol 1999;94(5):1197-9.

(106) Livermore DM. beta-Lactamases in laboratory and clinical resistance. Clin Microbiol Rev 1995;8(4):557-84.

(107) Jacoby GA, Archer GL. New mechanisms of bacterial resistance to antimicrobial agents. N Engl J Med 1991;324(9):601-12.

(108) Nikaido H. Multidrug efflux pumps of gram-negative bacteria. J Bacteriol 1996;178(20):5853-9.

(109) Ghuysen JM. Serine beta-lactamases and penicillin-binding proteins. Annu Rev Microbiol 1991;45:37-67.

(110) Massova I, Mobashery S. Kinship and diversification of bacterial penicillin-binding proteins and beta-lactamases. Antimicrob Agents Chemother 1998;42(1):1-17.

(111) Krishnamurthy P, Parlow MH, Schneider J, Burroughs S, Wickland C, Vakil NB, *et al*. Identification of a novel penicillin-binding protein from *Helicobacter pylori*. J Bacteriol 1999;181(16):5107-10.

(112) Tee W, Fairley S, Smallwood R, Dwyer B. Comparative evaluation of three selective media and a nonselective medium for the culture of *Helicobacter pylori* from gastric biopsies. J Clin Microbiol 1991;29(11):2587-9.

(113) Megraud F, Lehn N, Lind T, Bayerdorffer E, O'Morain C, Spiller R, *et al.* Antimicrobial susceptibility testing of *Helicobacter pylori* in a large multicenter trial: the MACH 2 study. Antimicrob Agents Chemother 1999;43(11):2747-52.

(114) Lang L, Garcia F. Comparison of E-test and disk diffusion assay to evaluate resistance of *Helicobacter pylori* isolates to amoxicillin, clarithromycin, metronidazole and tetracycline in Costa Rica. Int J Antimicrob Agents 2004;24(6):572-7.

(115) Sutton LD, Biedenbach DJ, Yen A, Jones RN. Development, characterization, and initial evaluations of S1. A new chromogenic cephalosporin for beta-lactamase detection. Diagn Microbiol Infect Dis 1995;21(1):1-8.

(116) Ani AE, Malu AO, Onah JA, Queiroz DM, Kirschner G, Rocha GA. Antimicrobial susceptibility test of *Helicobacter pylori* isolated from Jos, Nigeria. Trans R Soc Trop Med Hyg 1999;93(6):659-61.

(117) Realdi G, Dore MP, Piana A, Atzei A, Carta M, Cugia L, *et al*. Pretreatment antibiotic resistance in *Helicobacter pylori* infection: results of three randomized controlled studies. Helicobacter 1999;4(2):106-12.

(118) Destura RV, Labio ED, Barrett LJ, Alcantara CS, Gloria VI, Daez ML, *et al.* Laboratory diagnosis and susceptibility profile of *Helicobacter pylori* infection in the Philippines. Ann Clin Microbiol Antimicrob 2004;3:25.

(119) Smith AM, Klugman KP. Alterations in PBP 1A essential-for high-level penicillin resistance in *Streptococcus pneumoniae*. Antimicrob Agents Chemother 1998;42(6):1329-33.

(120) Du Plessis M, Bingen E, Klugman KP. Analysis of penicillin-binding protein genes of clinical isolates of *Streptococcus pneumoniae* with reduced susceptibility to amoxicillin. Antimicrob Agents Chemother 2002;46(8):2349-57.

(121) Di Guilmi AM, Mouz N, Andrieu JP, Hoskins J, Jaskunas SR, Gagnon J, *et al.* Identification, purification, and characterization of transpeptidase and glycosyltransferase domains of *Streptococcus pneumoniae* penicillin-binding protein 1a. J Bacteriol 1998;180(21):5652-9.

(122) Sanchez M, Vicente MF, Cercenado E, de Pedro MA, Gomez P, Moreno R, *et al.* Diversity among clinical isolates of penicillin-resistant *Streptococcus mitis*: indication for a PBP1-dependent way to reach high levels of penicillin resistance. Int Microbiol 2001;4(4):217-22.

(123) Ropp PA, Hu M, Olesky M, Nicholas RA. Mutations in *ponA*, the gene encoding penicillin-binding protein 1, and a novel locus, *penC*, are required for high-level chromosomally mediated penicillin resistance in *Neisseria gonorrhoeae*. Antimicrob Agents Chemother 2002;46(3):769-77.

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APPENDICES

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APPENDIX I

REAGENTS, MATERIALS AND INSTRUMENTS

A. REAGENTS

Absolute ethanol	(Merck, Germany)
Agarose	(Biorad, USA)
Brain heart infusion agar	(Oxoid, England)
EDTA	(Amresco, USA)
Ethidium bromide	(Amresco, USA)
Glacial acetic acid	(Merck, Germany)
Horse serum	(GibcoBRL, USA)
Miniral oil	(Sigma, USA)
Columbia agar base	(Oxoid, England)
Urea agar base	(BBL, U.S.A.)
Bacto agar	(Difco, USA)
NaCl	(Merck, Germany)
$NaHCO_3$	(Merck, Germany)
$Na_2HPO_4*2H_2O$	(Sigma, USA)
Tris	(Amresco, USA)

B. MATERIALS

Anaerobic jar	(BBL, USA)
Gas pack	(Oxoid, England)

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C. INSTRUMENTS

Water bath	(Memmert, USA)
Perkin Elmer GeneAmp PCR system 9600	(Perkin Elmer, USA)
Camera Gel Doc™ MZL	(BIO-RAD, USA)
Incubator	(BIO-RAD, USA)
Microcentrifuge	(Eppendorf, USA)
Spectrophotometer	(BIO-RAD, USA)

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APPENDIX II

MEDIA, SOLUTION AND IDENTIFICATION PROCEDURES

1. Columbia agar with 7 % sheep blood

Columbia agar base	39 g/L
Horse serum	70 ml/L
Sheep blood	70 ml/L
Distilled water	860 ml

The medium was sterillized by autoclaving at 121^{o}C, 15 pounds/inch^{2} pressure, for 15 minutes. The sterile medium was cooled to 45^{o}C to 50^{o}C. Add blood and horse serum after cooling base medium. Dispense 20 ml per petri dish. Cool and store at 4^{o}C until used.

Do not add any heat labile components (Sheep blood or antibiotic solutions) to the sterillizer.

2. Columbia agar with 7 % Sheep blood and antibiotics

Columbia agar base	39 g/L
Horse serum	70 ml/L
Sheep blood	70 ml/L
Vancomycin (1 ml of stock)	10 mg/L
Trimethoprim (0.5 ml of stock)	5 mg/L
Cefsoludin (0.5 ml of stock)	5 mg/L
Amphotericin B (0.5 ml of stock)	5 mg/L
Distilled water	860 ml

The medium was sterillized by autoclaving at 121^{o}C, 15 pounds/inch^{2} pressure, for 15 minutes. The sterile medium was cooled to 45^{o}C to 50^{o}C. Add blood, horse serum and antibiotic solution after cooling base medium. Dispense 20 ml per petri dish. Cool and store at 4^{o}C until used.

Do not add any heat labile components (Sheep blood or antibiotic solutions) to the sterillizer.

3. Antibiotic solution preparation

Vancomycin, final concentration 10 mg/L

- Prepare a stock solution; dissolve 0.028 g in 5.78 ml distilled water

Cefsoludin, final concentration 5 mg/L

- Prepare a stock solution, dissolve 0.014 g in 2.82 ml distilled water.

Trimethoprim, final concentration 5 mg/L

- Prepare a stock solution, dissolve 0.018 g in 3.7 ml distilled water.

Amphotericin, final concentration 5 mg/L

- Prepare a stock solution, dissolve 0.014 g in 2.84 ml distilled water.

4. Urease test

Solution A: for	20 ml
Urea agar base (BBL)	2.9 g
Urea	4 g
Distilled water	20 ml

Dissolve in 20 ml of distilled water. Adjust to pH 6.2 Sterilize by filtration (use a 0.22 μm filter).

Solution B: for	80 ml
Bacto agar (Difco)	0.5 g
Distilled water	80 ml

Add the ingredient to 80 ml of distilled water; heat with stirring until the agar is dissolved. Sterilize by autoclaving at 121^{o}C, 15 ponds/inch^{2} pressure, for 15 minutes. Mix solution A, 20 ml with solution B, 80 ml. Aliquot into sterile 1.5 microtube (1 ml/tube). Test the sterility of Urease medium by incubate tubes at 37^{o}C for 24 hours. Store tubes in refrigerator at 4^{o}C until used.

5. Brain heart infusion with 20 % glycerol

Brain heart infusion	37 g/L
Glycerol	200 ml
Distilled water	800 ml

Sterilize by autoclaving at 121^{o}C, 15 pounds/inch^{2} pressure, for 15 minutes. Aliquot into sterile screw cap tubes (1 ml/tube). Store tubes in refrigerator at 4^{o}C until used.

6. Sterile saline solution

Sodium Chloride	8.5 g/L
Distilled water	1 L

Sterilze by autoclaving at 121^{o}C, 15 pounds/inch^{2} pressure, for minutes. Store at room temperature.

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APPENDIX III

REAGENTS AND PREPARATION

1. 5x Tris-borate buffer (TBE)

Tris base	54 g/L
Boric acid	27.5 g/L
0.5 M EDTA (pH 8.0)	20 ml

Adjust volume to 1 liter with distilled water. The solution was mixed and sterilized by autoclaving at 121^{o}C for 15 min.

2. 0.5 M EDTA (pH 8.0)

Disodium ethylene diamine tetra-acelate $2H_2O$	186.1 g/L
Distilled water	1 L

Adjust pH to 8.0 and volume to 1 liter. Store at room temperature for no longer than 1 year.

3. 10x TE buffer

Tris	12.11 g/L
0.5 M EDTA	20 ml

Adjust to pH 8.0 by adding conc. HCl. Adjust volume to 1,000 ml and sterilized by autoclaving at 121^{o}C for 15 min.

4. 1 % Agarose gel

Agarose	0.2 g
1x TBE	20 ml

Dissolve by heating in microwave oven and occasional mix unit no granules of agarose are visible.

5. 5x Loading buffer 100 ml

Tris HCl	0.6 g
EDTA	1.68 g
SDS	0.5 g

Bromphenol Blue	0.1 g
Sucrose	40 g

Adjust volume to 100 ml with distilled water. Mix the solution, aliquot into 1.5 microtubes and store at 4^{o}C.

Reagent for DNA Extraction

Protease K

Reconstituted of protease K (lyophilized) with 1.25 ml protease solvent, stored at –20^{o}C

1.2 Buffer AL (Ready to used)

1.3 Buffer AW1

Buffer AW1 is supplied as a concentrate. Before using for the first time, add the 25 ml of ethanol (96-100%) to buffer AW1 concentrate as indicated on the bottle.

1.4 Buffer AW2

Buffer AW2 is supplied as a concentrate. Before using for the first time, add the 30 ml of ethanol (96-100%) to buffer AW2 concentrate as indicated on the bottle.

1.5 Buffer AE (Ready to used)

Reagent for PCR product purification

Buffer PB (Ready to used)

Buffer PE

Buffer PE is supplied as a concentrate. Before using for the first time, add the 55 ml of ethanol (96-100%) to buffer PE concentrate as indicated on the bottle.

APPENDIX IV

Table 7 *H. pylori* isolates, demographic information of patients and amoxicillin MICs (μg/ml) .

Number	*H. pylori* strains	Age	Sex*	Diagnosis*	MIC by E-test (μg/ml)
1	HP-826	63	F	NUD	< 0.016
2	HP-827	29	F	NUD	< 0.016
3	HP-846	62	F	GU	< 0.016
4	HP-847	26	F	NUD	< 0.016
5	HP-858	56	F	NUD	< 0.016
6	HP-870	64	F	NUD	0.047
7	HP-874	28	M	NUD	< 0.016
8	HP-876	51	M	NUD	< 0.016
9	HP-890	70	M	GU	0.016
10	HP-903	60	F	NUD	0.016
11	HP-904	38	F	NUD	< 0.016
12	HP-911	65	M	NUD	< 0.016
13	HP-927	69	F	GU	0.016
14	HP-976	89	M	GU	< 0.016
15	HP-977	67	F	NUD	< 0.016
16	HP-989	28	F	NUD	< 0.016
17	HP-991	54	M	GU	< 0.016
18	HP-992	36	F	NUD	< 0.016
19	HP-994	63	M	GU	< 0.016
20	HP-1001	32	F	NUD	< 0.016
21	HP-1002	73	M	DU	< 0.016
22	HP-1007	49	M	DU	0.016
23	HP-1014	65	M	NUD	0.016
24	HP-1027	42	F	NUD	< 0.016
25	HP-1028	23	F	NUD	0.25
26	HP-1029	79	M	NUD	0.016

Table 7 *H. pylori* isolates, demographic information of patients and amoxicillin MICs (μg/ml) (Continued).

Number	*H. pylori* strains	Age	Sex*	Diagnosis*	MIC by E-test (μg/ml)
27	HP-1037	64	F	NUD	< 0.016
28	HP-1038	26	F	NUD	< 0.016
29	HP-1045	34	M	NUD	< 0.016
30	HP-1051	60	F	NUD	< 0.016
31	HP-1052	45	F	NUD	< 0.016
32	HP-1059	61	F	NUD	<0.016
33	HP-1076	45	M	GU	0.016
34	HP-1081	53	F	NUD	0.016
35	HP-1101	84	F	GU	0.016
36	HP-1107	53	M	NUD	<0.016
37	HP-1111	38	F	NUD	< 0.016
38	HP-1112	36	F	NUD	< 0.016
39	HP-1123	71	M	NUD	<0.016
40	HP-1129	40	M	GU	< 0.016
41	HP-1135	39	F	NUD	< 0.016
42	HP-1140	54	F	NUD	< 0.016
43	HP-1142	38	M	DU	0.016
44	HP-1144	74	M	NUD	0.75
45	HP-1146	35	M	GU	< 0.016
46	HP-1162	53	M	GU	< 0.016
47	HP-1163	60	F	GU	0.016
48	HP-1164	42	F	NUD	< 0.016
49	HP-1173	52	M	GU	< 0.016
50	HP-1174	60	F	GU	< 0.016

* M, man; F, female; NUD, non-ulcer dyspepsia; GU, gastric ulcer; and DU, duodenal ulcer

Figure 11 Alignment of amino acid sequences of PBP1.

```
           251                                                        300
    26695  YNQTSTQNIA PYVVDEVLKQ LDQLDGLKTQ GYTIKLTIDL DYQRLALESL
      HPC  YNQTSTQNIA PYVVDEVLKQ LDQLDGLKTQ GYTIKLTIDL DYQRLALESL
  HP-1163  YNQTSTQNIA PYVVDEVLKQ LDQLDGLKTQ GYTIKLTIDL DYQRLALESL
  HP-1174  YNQTSTQNIA PYVVDEVLKQ LDQLDGLKTQ GYTIKLTIDL DYQRLALESL
  HP-1144  YNQTSTQNIA PYVVDEVLKQ LDQLDGLKTQ GYTIKLTIDL DYQRLALESL
   HP-870  YNQTSTQNIA PYVVDEVLKQ LDQLDGLKTQ GYTIKLTIDL DYQRLALESL
  HP-1028  YNQTSTQNIA PYVVDEVLKQ LDQLDGLKTQ GYTIKLTIDL DYQRLALESL
Consensus  YNQTSTQNIA PYVVDEVLKQ LDQLDGLKTQ GYTIKLTIDL DYQRLALESL

           301                                                        350
    26695  RFGHQKILEK IAKEKPKTNA SNEDEDNLNA SMIVTDTSTG KILALVGGID
      HPC  RFGHQKILEK IAKEKPKTNA SDEDEDNLNA SMIVTDTSTG KILALVGGID
  HP-1163  RFGHQKILEK IAKEKPKTNA SDEDEDNLNA SMIVTDTSTG KILALVGGID
  HP-1174  RFGHQKILEK IAKEKPKTNA SNEYEDNLNA SMIVTDTSTG KILALVGGID
  HP-1144  RFGHQKILEK IAKEKPKTNA SNEYEDNLNA SMIVTDTSTG KILALVGGID
   HP-870  RFGHQKILEK IAKEKPKTNA SNEDEDNLNA SMIVTDTSTG KILALVGGID
  HP-1028  RFGHQKILEK IAKEKPKTNA SNEDEDNLNA SIIVTDTSTG KILALVGGID
Consensus  RFGHQKILEK IAKEKPKTNA S#EdEDNLNA SmIVTDTSTG KILALVGGID

           351                                                        400
    26695  YKKSAFNRAT QAKRQFGSAI KPFVYQIAFD NGYSTTSKIP DTARNFENGN
      HPC  YKKSAFNRAT QAKRQFGSAI KPFVYQIAFD NGYSTTSKIP DTARNFENGN
  HP-1163  YKKSAFNRAT QAKRQFGSAI KPFVYQIAFD NGYSTTSKIP DAARNFENGN
  HP-1174  YKKSAFNRAT QAKRQFGSAI KPFVYQIAFD NGYSTTSKIP DTARNFENGN
  HP-1144  YKKSAFNRAT QAKRQFGSAI KPFVYQIAFD NGYSTTSKIP DTARNFENGN
   HP-870  YKKSAFNRAT QAKRQFGSAI KPFVYQIAFD NGYSTTSKIP DTARNFENGN
  HP-1028  YEKSAFNRAT QAKRQFGSAI KPFLYQIAFD NGYSTTSKIP DTARNFENG-
Consensus  YkKSAFNRAT QAKRQFGSAI KPFvYQIAFD NGYSTTSKIP DtARNFENGn

           401                                                        450
    26695  YSKNSEQNHA WHPSNYSRKF LGLVTLQEAL SHSLNLATIN LSDQLGFEKI
      HPC  YSKNSEQNHA WHPSNYSRKF LGLVTLQEAL SHSLNLATIN LSDQLGFEKI
  HP-1163  YSKNSVQSHA WHPSNYSRKF LGLVTLQEAL SLSLNLATIN LSDQLGFEKI
  HP-1174  YSKNSEQNHA WHPSNYSRKF LGLVTLQEAL SHSLNLATIN LSDQLGFEKI
  HP-1144  YSKNSEQNHA WHPSNYSRKF LGLVTLQEAL SHSLNLATIN LSDQLGFEKI
   HP-870  YSKNSEQNHA WHPSNYSRKF LGLVTLQEAL SHSLNLATIN LSDQLGFEKI
  HP-1028  YSKNSEQNHA WHPRNYSRKF LGLVTLQEAL SHSLNLATIN LSDQLGFEKI
Consensus  YSKNSeQnHA WHPsNYSRKF LGLVTLQEAL ShSLNLATIN LSDQLGFEKI

           451                                                        500
    26695  YQSLSDMGFK NLPKDLSIVL GSFAISPIDA AEKYSLFSNY GTMLKPMLIE
      HPC  YQSLSDMGFK NLPKDLSIVL GSFAISPIDA AEKYSLFSNY GTMLKPMLIE
  HP-1163  YQSLSDMGFK NLPKDLSIVL GSFAISPIDA AEKYSLFSNY GTMLKPMLIE
  HP-1174  YQSLSDMGFK NLPKDLSIVL GSFAISPIEA AEKYSLFSNY GTMLKPMLIE
  HP-1144  YQSLSDMGFK NLPKDLSIVL GSFAISPIEA AEKYSLFSNY GTMLKPMLIE
   HP-870  YQSLSDMGFK NLPKDLSIGL GSFAISPIEA AEKYSLFSNY GTMLKPMLIE
  HP-1028  YQSLSDMGFK NLPKDLSIVL GSFAISPIEA AEKYSLFSNY GTMLKPMLIE
Consensus  YQSLSDMGFK NLPKDLSIvL GSFAISPI#A AEKYSLFSNY GTMLKPMLIE
```

```
           501                                                  550
    26695  SITNQQNDVK TFTPMETKKI TSKEQAFLTL SVLMDAVENG TGSLARIKGL
      HPC  SITNQQNDVK TFTPMETKKI TSKEQAFLTL SVLINAVENG TGRLARTKGL
  HP-1163  SITNQQNDVK TFTPMETKKI TSKEQAFLTL SVLINAVENG TGRLARTKGL
  HP-1174  SITDQQNNIK TFTPIETKKI TSKEQAFLTL SVLMNAVENG TGNLARIKGL
  HP-1144  SITDQQNNIK TFTPIETKKI TSKEQAFLTL SVLMNAVENG TGNLARIKGL
   HP-870  SITDQQNDVK TFTPMETKKI TSKEQAFLTL SVLMNAVENG TGSLARIKGL
  HP-1028  SITDQQNDVK TFTPIETKKI TSKEQAFLTL SVLMNAVENG TGSLARIKGL
Consensus  SIT#QQN#!K TFTPmETKKI TSKEQAFLTL SVLm#AVENG TGsLARiKGL

           551                                                  600
    26695  EIAGKTGTSN NNIDAWFIGF TPTLQSVIWF GRDDNTPISK GATGGVVSAP
      HPC  EIAGKTGTSN NNIDAWFIGF TPTLQSVIWF GRDDNTPISK GATGGVVSAP
  HP-1163  EIAGKTGTSN NNIDAWFIGF TPTLQSVIWF GRDDNTPISK GATGGVVSAP
  HP-1174  EIAGKTGTSN NNIDAWFIGF TPTLQSVIWF GRDDNTPISK GATGGVVSAP
  HP-1144  EIAGKTGTSN NNIDAWFIGF TPTLQSVIWF GRDDNTPISK GATGGVVSAP
   HP-870  EIAGKTGTSN NNIDAWFIGF TPTLQSVIWF GRDDNTPIGK GAAGSVVSAP
  HP-1028  EIAGKSGTSN NNIDAWFIGF TPTLQSVIWF GRDDNTPIGK GATGGVVSAP
Consensus  EIAGKtGTSN NNIDAWFIGF TPTLQSVIWF GRDDNTPIsK GAtGgVVSAP


           601                                                  650
    26695  VYSYFMRNIL AIEPSLKRKF DVPKGLRKEI VDKIPYYSTP NSITPTPKRT
      HP-  VYSYFMRNIL AIEPSLKRKF DVPKGLRKEI VDKIPYYSTP NSITPTPKRT
  HP-1163  VYSYFMRNIL AIEPSLKRKF DVPKGLRKEI VDKIPYYSTP NSITPTPKRT
  HP-1174  VYSYFMRNIL AIEPSLKRKF DVPKGLRKEI VDKIPYYSTP NSITPTPQKT
  HP-1144  VYSYFMRNIL AIEPSLKRKF DVPKGLRKEI VDKIPYYSTP NSITPTPQKT
   HP-870  VYSYFMRNIL AIEPSLKRKF DVPKGLRKEI VDKIPYYSTP NSITPTPQKT
  HP-1028  VYSYFMRNIL SIEPSLKRKF DVPKGLRKEI VDKIPYYSTP NSITPTPKRT
Consensus  VYSYFMRNIL aIEPSLKRKF DVPKGLRKEI VDKIPYYSTP NSITPTPkrT

           651
    26695  DDSEERLLF
      HP-  DDSEERLLF
  HP-1163  DDSEERLLF
  HP-1174  DDSEERLLF
  HP-1144  DDSEERLLF
   HP-870  DDGKEPLLF
  HP-1028  DDSEEPLLF
Consensus  DDseErLLF
```

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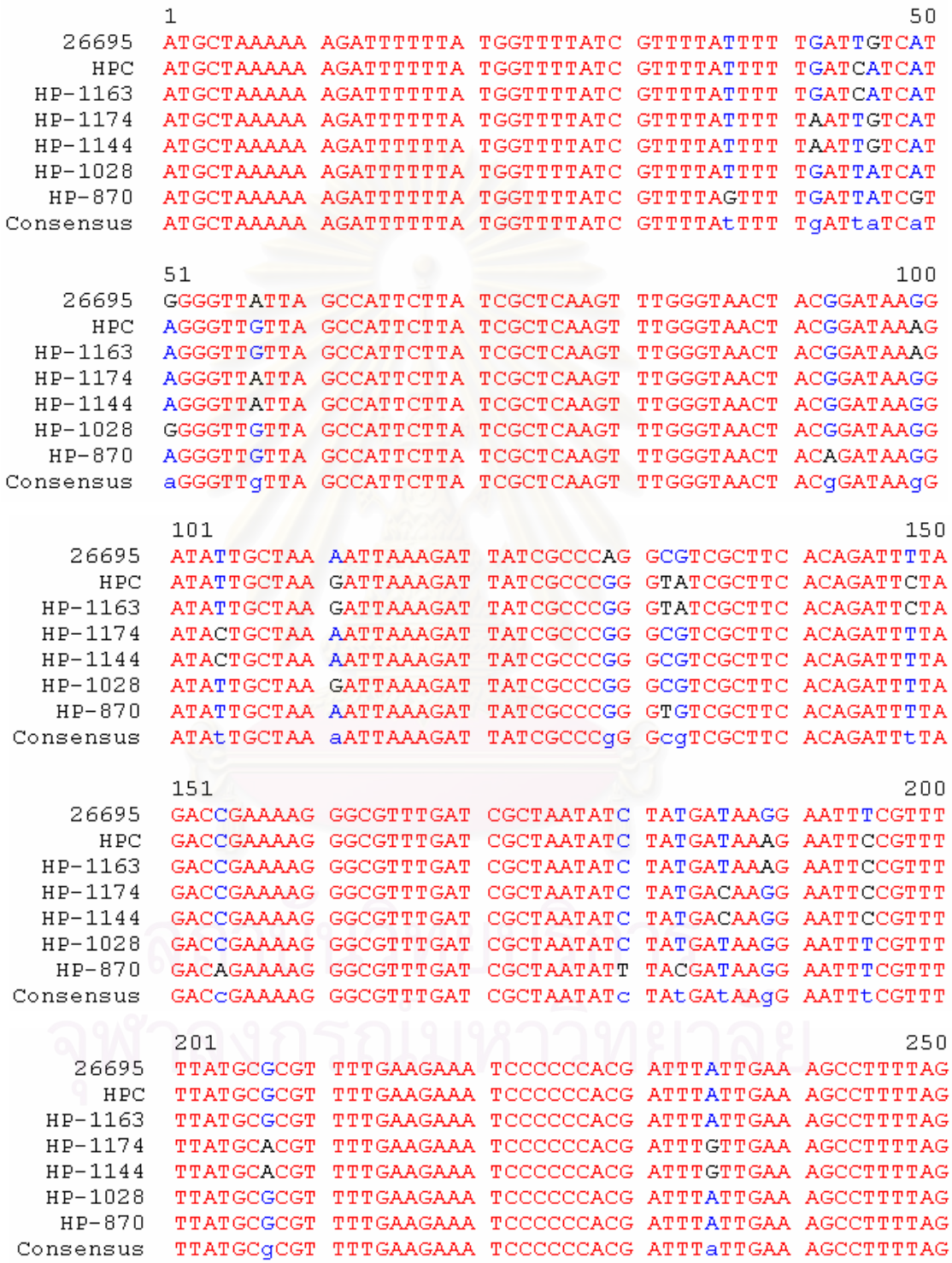
Abbreviation: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, trytophan; Y, tyrosine; 26695, *H. pylori* 26695; HP, *H. pylori*; HPC, *H. pylori* NCTC 11637

Figure 12 Alignment of nucleotide sequences of *pbp1* gene.



```
          251                                                        300
   26695  CGGTAGAAGA CACCCTCTTT TTTGAACATG GGGGGATCAA TTTAGACGCT
     HPC  CGGTAGAAGA CACCCTCTTT TTTGAACATG GGGGGATCAA TTTAGACGCT
 HP-1163  CGGTAGAAGA CACCCTCTTT TTTGAACATG GGGGGATCAA TTTAGACGCT
 HP-1174  CGGTAGAAGA CACCCTCTTT TTTGAACATG GGGGGATCAA TTTAGACGCT
 HP-1144  CGGTAGAAGA CACCCTCTTT TTTGAACATG GGGGGATCAA TTTAGACGCT
 HP-1028  CGGTAGAAGA CACCCTCTTT TTTGAGCATG GGGGGATCAA TTTAGACGCT
  HP-870  CAGTAGAAGA CACCCTCTTT TTTGAGCATG GGGGGATCAA TTTAGACGCT
Consensus  CgGTAGAAGA CACCCTCTTT TTTGAaCATG GGGGGATCAA TTTAGACGCT

          301                                                        350
   26695  ATCATGCGCG CTATGATTAA AAACGCTAAA AGCGGTCGTT ACACCGAGGG
     HPC  ATCATGCGCG CTATGATTAA AAACGCTAAA AGCGGTCGTT ACACTGAAGG
 HP-1163  ATCATGCGCG CTATGATTAA AAACGCTAAA AGCGGTCGTT ACACTGAAGG
 HP-1174  ATCATGCGCG CTATGATTAA AAATGCTAAA AGCGGTCGTA CCACCGAGGG
 HP-1144  ATCATGCGCG CTATGATTAA AAATGCTAAA AGCGGTCGTT ACACCGAGGG
 HP-1028  ATCATGCGCG CTATGATTAA AAACGCTAAA AGCGGTCGTT ACACTGAAGG
  HP-870  ATCATGCGTG CTATGATTAA AAACGCTAAA AGCGGTCGTT ACACCGAGGG
Consensus  ATCATGCGcG CTATGATTAA AAAcGCTAAA AGCGGTCGTt aCACcGAgGG

          351                                                        400
   26695  GGGTAGCACC CTAACCCAAC AATTCGTTAA AAACATGGTG CTCACACGAG
     HPC  GGGCAGCACT CTAACCCAAC AACTCGTTAA AAACATGGTG CTCACGCGAG
 HP-1163  GGGCAGCACT CTAACCCAAC AACTCGTTAA AAACATGGTG CTCACGCGAG
 HP-1174  GGGTAGCACC CTAACCCAAC AACTCGTTAA AAACATGGTG CTCACACGAG
 HP-1144  GGGTAGCACC CTAACCCAAC AACTCGTTAA AAACATGGTG CTCACACGAG
 HP-1028  GGGTAGCGCC CTAACCCAAC AACTCGTTAA AAACATGGTG CTCACACGAG
  HP-870  GGGTAGCACC CTAACCCAAC AACTCGTTAA AAACATGGTG CTCACACGAG
Consensus  GGGtAGCaCc CTAACCCAAC AAcTCGTTAA AAACATGGTG CTCACaCGAG

          401                                                        450
   26695  AAAAAACCCT AACCAGAAAA CTCAAAGAAG CGATCATTTC TATACGCATT
     HPC  AAAAAACATT AACCAGAAAA CTCAAAGAAG CTATCATTTC TTTACGCATT
 HP-1163  AAAAAACATT AACCAGAAAA CTCAAAGAAG CTATCATTTC TTTACGCATT
 HP-1174  AAAAAACATT AACCAGAAAA CTCAAAGAAG CTATCATTTC TTTACGCATT
 HP-1144  AAAAAACATT AACCAGAAAA CTCAAAGAAG CTATCATTTC TTTACGCATT
 HP-1028  AAAAAACGCT AACCAGAAAA CTCAAAGAAG CGATCATTTC CATACGCATT
  HP-870  AAAAAACCCT AACCAGAAAA CTCAAAGAAG CTATCATTTC CATACGCATT
Consensus  AAAAAAC.cT AACCAGAAAA CTCAAAGAAG CtATCATTTC taTACGCATT

          451                                                        500
   26695  GAAAAAGTCT TAAGCAAAGA AGAAATTTTA GAGCGTTATT TGAACCAAAC
     HPC  GAAAAAGTCT TAAGCAAAGA AGAAATTTTA GAGCGTTATT TGAACCAAAC
 HP-1163  GAAAAAGTCT TAAGCAAAGA AGAAATTTTA GAGCGTTATT TGAACCAAAC
 HP-1174  GAAAAAGTCT TAAGCAAAGA AGAAATTTTA GAGCGTTATT TGAACCAAAC
 HP-1144  GAAAAAGTCT TAAGCAAAGA AGAAATTTTA GAGCGTTATT TGAACCAAAC
 HP-1028  GAAAAAGTCT TAAGCAAAGA AGAAATTTTA GAGCGTTATT TGAACCAAAC
  HP-870  GAAAAAGTCT TAAGCAAAGA AGAAATTTTA GAGCGCTATT TGAACCAAAC
Consensus  GAAAAAGTCT TAAGCAAAGA AGAAATTTTA GAGCGtTATT TGAACCAAAC
```

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           501                                                  550
    26695  TTTTTTTGGG CATGGGTATT ATGGCGTGAA AACCGCAAGT TTAGGGTATT
      HPC  TTTTTTTGGG CATGGGTATT ATGGCGTGAA AACCGCAAGT TTAGGGTATT
   HP-1163 TTTTTTTGGG CATGGGTATT ATGGCGTGAA AACCGCAAGT TTAGGGTATT
   HP-1174 TTTTTTTGGG CATGGGTATT ATGGCGTGAA AACCGCAAGT TTAGGGTATT
   HP-1144 TTTTTTTGGG CATGGGTATT ATGGCGTGAA AACCGCAAGT TTAGGGTATT
   HP-1028 TTTTTTTGGG CATGGGTATT ATGGCGTGAA AACCGCAAGT TTAGGGTATT
    HP-870 TTTTTTTGGG CATGGGTATT ATGGCGTCAA AACCGCAAGT TTAGGGTATT
 Consensus TTTTTTTGGG CATGGGTATT ATGGCGTgAA AACCGCAAGT TTAGGGTATT

           551                                                  600
    26695  TTAAAAAACC CCTTGACAAA CTCACGCTTA AAGAAATCAC CATGCTAGTC
      HPC  TTAAAAAACC CCTTGACAAA CTCACGCTTA AAGAAATCAC CATGTTAGTC
   HP-1163 TTAAAAAACC CCTTGACAAA CTCACGCTTA AAGAAATCAC CATGTTAGTC
   HP-1174 TTAAAAAACC CCTTGACAAA CTCACGCTTA AAGAAATCAC CATGTTAGTC
   HP-1144 TTAAAAAACC CCTTGACAAA CTCACGCTTA AAGAAATCAC CATGTTAGTC
   HP-1028 TTAAAAAACC CCTTGACAAA CTCACGCTTA AAGAAATCAC CATGTTAGTC
    HP-870 TTAAAAAACC CCTTGACAAA CTCACGCTTA AAGAAATCAC CATGTTAGTC
 Consensus TTAAAAAACC CCTTGACAAA CTCACGCTTA AAGAAATCAC CATGtTAGTC

           601                                                  650
    26695  GCCTTGCCTA GGGCTCCGAG TTTTTATGAT CCTACCAAAA ATTTAGAATT
      HPC  GCCTTGCCTA GGGCTCCGAG TTTTTATGAT CCTACCAAAA ATTTAGAATT
   HP-1163 GCCTTGCCTA GGGCTCCGAG TTTTTATGAT CCTACCAAAA ATTTAGAATT
   HP-1174 GCCTTGCCCA GAGCTCCGAG TTTTTATGAC CCTACCAAAA ATTTAGAATT
   HP-1144 GCCTTGCCCA GAGCTCCGAG TTTTTATGAC CCTACCAAAA ATTTAGAATT
   HP-1028 GCCTTGCCTA GGGCTCCGAG TTTTTATGAC CCTACCAAAA ATTTAGAATT
    HP-870 GCCTTGCCTA GGGCTCCAAG CTTTTATGAC CCTACCAAAA ATTTAGAATT
 Consensus GCCTTGCCtA GgGCTCCgAG tTTTTATGAc CCTACCAAAA ATTTAGAATT

           651                                                  700
    26695  TTCACTCTCT AGGGCTAATG ATATTTTAAG GCGGTTGTAT TCTTTAGGCT
      HPC  TTCACTCTCT AGAGCTAATG ATATTTTAAG GCGGTTGTAT TCTTTAGGCT
   HP-1163 TTCACTCTCT AGAGCTAATG ATATTTTAAG GCGGTTGTAT TCTTTAGGCT
   HP-1174 TTCACTTTCT AGGGCTAATG ATATTTTAAG GCGGTTGTAT TCTTTGGGTT
   HP-1144 TTCACTTTCT AGGGCTAATG ATATTTTAAG GCGGTTGTAT TCTTTGGGTT
   HP-1028 TTCACTCTCT AGGGCTAATG ATATTTTAAG GCGGTTGTAT TCTTTGGGTT
    HP-870 TTCACTCTCT AGGGCTAATG ATATTTTAAG GCGGTTGTAT TCTTTAGGCT
 Consensus TTCACTcTCT AGgGCTAATG ATATTTTAAG GCGGTTGTAT TCTTTaGGcT

           701                                                  750
    26695  GGATTTCTTC TAACGAGCTC AAAGGCGCTC TCAATGAAGT GCCAATTGTT
      HPC  GGATTTCTTC TAACGAGCTC AAAGGCGCTC TCAATGAAGT GCCAATCGTC
   HP-1163 GGATTTCTTC TAACGAGCTC AAAGGCGCTC TCAATGAAGT GCCAATCGTC
   HP-1174 GGATCTCTTC TAACGAGCTC AAAGGCGCTC TCAATGAAGT GCCAATCGTC
   HP-1144 GGATCTCTTC TAACGAGCTC AAAGGCGCTC TCAATGAAGT GCCAATCGTC
   HP-1028 GGATCTCTTC TAACGAGCTC AAAGGCGCTC TCAATGAAGT GCCAATCGTC
    HP-870 GGATTTCTTC TAACGAGCTC AAATCCTCTC TCAATGAAGT GCCAATCGTC
 Consensus GGATtTCTTC TAACGAGCTC AAAggCgCTC TCAATGAAGT GCCAATcGTc
```

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           751                                                       800
    26695  TATAACCAAA CCTCCACGCA AAACATCGCC CCCTATGTCG TAGATGAAGT
      HPC  TATAACCAAA CTTCCACGCA AAATATCGCT CCCTATGTCG TGGATGAAGT
   HP-1163 TATAACCAAA CTTCCACGCA AAATATCGCT CCCTATGTCG TGGATGAAGT
   HP-1174 TATAACCAAA CTTCCACGCA AAATATCGCC CCCTATGTCG TGGATGAAGT
   HP-1144 TATAACCAAA CTTCCACGCA AAATATCGCC CCCTATGTCG TGGATGAAGT
   HP-1028 TATAACCAAA CTTCCACGCA AAATATCGCC CCCTATGTCG TGGATGAAGT
    HP-870 TATAACCAAA CTTCCACGCA AAATATCGCT CCCTATGTCG TGGATGAAGT
 Consensus TATAACCAAA CtTCCACGCA AAAtATCGCc CCCTATGTCG TgGATGAAGT

           801                                                       850
    26695  GTTGAAGCAA TTGGATCAAT TAGACGGGTT AAAAACTCAA GGCTATACCA
      HPC  GTTGAAGCAA TTGGATCAAT TAGACGGGTT AAAAACTCAA GGCTATACCA
   HP-1163 GTTGAAGCAA TTGGATCAAT TAGACGGGTT AAAAACTCAA GGCTATACCA
   HP-1174 GTTGAAGCAA TTGGATCAAT TAGACGGGTT AAAAACTCAA GGCTATACCA
   HP-1144 GTTGAAGCAA TTGGATCAAT TAGACGGGTT AAAAACTCAA GGCTATACCA
   HP-1028 GTTGAAGCAA TTGGATCAAT TAGACGGGTT AAAAACTCAA GGCTATACCA
    HP-870 GTTGAAGCAA TTGGATCAAT TAGACGGGTT AAAAACTCAA GGCTATACCA
 Consensus GTTGAAGCAA TTGGATCAAT TAGACGGGTT AAAAACTCAA GGCTATACCA

           851                                                       900
    26695  TAAAGCTCAC GATAGATTTG GATTACCAAC GCTTAGCGTT AGAGTCCTTG
      HPC  TAAAGCTCAC GATAGATTTG GATTACCAAC GCTTAGCGTT AGAGTCCTTG
   HP-1163 TAAAGCTCAC GATAGATTTG GATTACCAAC GCTTAGCGTT AGAGTCCTTG
   HP-1174 TCAAGCTCAC GATAGATTTG GATTACCAAC GCTTAGCGTT AGAGTCTTTG
   HP-1144 TCAAGCTCAC GATAGATTTG GATTACCAAC GCTTAGCGTT AGAGTCTTTG
   HP-1028 TCAAGCTCAC GATAGATTTG GATTACCAAC GCTTAGCGTT AGAGTCTTTG
    HP-870 TAAAACTCAC GATAGATTTG GATTACCAAC GCTTGGCGTT AGAGTCCTTG
 Consensus TaAAgCTCAC GATAGATTTG GATTACCAAC GCTTaGCGTT AGAGTCcTTG

           901                                                       950
    26695  CGTTTTGGGC ATCAAAAAAT CTTAGAAAAA ATCGCTAAAG AAAAGCCAAA
      HPC  CGTTTTGGGC ATCAAAAAAT CTTAGAAAAA ATCGCTAAAG AAAAGCCAAA
   HP-1163 CGTTTTGGGC ATCAAAAAAT CTTAGAAAAA ATCGCTAAAG AAAAGCCAAA
   HP-1174 CGTTTTGGGC ACCAAAAAAT CTTAGAAAAA ATCGCTAAAG AAAAGCCAAA
   HP-1144 CGTTTTGGGC ACCAAAAAAT CTTAGAAAAA ATCGCTAAAG AAAAGCCAAA
   HP-1028 CGTTTTGGGC ACCAAAAAAT CTTAGAAAAA ATCGCTAAAG AAAAGCCAAA
    HP-870 CGTTTTGGGC ATCAAAAAAT CTTAGAAAAA ATCGCTAAAG AAAAGCCAAA
 Consensus CGTTTTGGGC AtCAAAAAAT CTTAGAAAAA ATCGCTAAAG AAAAGCCAAA


           951                                                      1000
    26695  AACTAACGCG TCTAATGAAG ATGAAGACAA CTTAAACGCT AGCATGATCG
      HPC  AACTAACGCC TCTGATGAAG ATGAAGACAA CTTGAACGCC AGCATGATAG
   HP-1163 AACTAACGCC TCTGATGAAG ATGAAGACAA CTTGAACGCC AGCATGATAG
   HP-1174 AACTAACGCA TCTAATGAAT ATGAAGACAA CTTAAACGCT AGCATGATCG
   HP-1144 AACTAACGCA TCTAATGAAT ATGAAGACAA CTTAAACGCT AGCATGATCG
   HP-1028 AACTAACGCA TCTAATGAAG ATGAAGATAA CTTGAACGCT AGCATAATCG
    HP-870 AACTAACGCC TCTAATGAAG ATGAAGACAA CTTGAACGCC AGCATGATAG
 Consensus AACTAACGC. TCTaATGAAg ATGAAGAcAA CTTgAACGCt AGCATgATcG
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           1001                                                1050
    26695  TTACAGACAC GAGCACCGGT AAGATTTTAG CTTTAGTGGG GGGGATTGAT
      HPC  TTACAGACAC GAGCACCGGT AAGATTTTAG CTTTAGTGGG GGGGATTGAT
  HP-1163  TTACAGACAC GAGCACCGGT AAGATTTTAG CTTTAGTGGG GGGGATTGAT
  HP-1174  TTACAGACAC GAGCACCGGT AAGATTTTAG CTTTAGTGGG GGGGATTGAT
  HP-1144  TTACAGACAC GAGCACCGGT AAGATTTTAG CTTTAGTGGG GGGGATTGAT
  HP-1028  TTACAGACAC GAGTACCGGT AAGATTTTAG CTTTAGTGGG GGGGATTGAT
   HP-870  TTACAGACAC AAGCACCGGT AAGATTTTAG CTCTAGTGGG GGGGATTGAT
Consensus  TTACAGACAC gAGcACCGGT AAGATTTTAG CTtTAGTGGG GGGGATTGAT

           1051                                                1100
    26695  TATAAAAAAA GCGCTTTCAA TCGCGCCACG CAAGCCAAAC GGCAGTTTGG
      HPC  TATAAAAAAA GCGCTTTCAA TCGCGCCACG CAAGCCAAAC GGCAGTTTGG
  HP-1163  TATAAAAAAA GCGCTTTCAA TCGCGCCACG CAAGCCAAAC GGCAGTTTGG
  HP-1174  TATAAAAAAA GCGCTTTCAA TCGCGCCACG CAAGCCAAAC GGCAGTTTGG
  HP-1144  TATAAAAAAA GCGCTTTCAA TCGCGCCACG CAAGCCAAAC GGCAGTTTGG
  HP-1028  TATGAAAAAA GCGCTTTCAA TCGCGCCACG CAAGCCAAAC GGCAATTTGG
   HP-870  TATAAAAAAA GCGCTTTCAA TCGCGCCACG CAAGCCAAAC GGCAATTTGG
Consensus  TATaAAAAAA GCGCTTTCAA TCGCGCCACG CAAGCCAAAC GGCAgTTTGG

           1101                                                1150
    26695  GAGCGCGATA AAGCCTTTTG TGTATCAGAT CGCTTTTGAT AATGGCTATT
      HPC  GAGCGCGATA AAGCCTTTTG TGTATCAAAT CGCTTTTGAT AATGGCTATT
  HP-1163  GAGCGCGATA AAGCCTTTTG TGTATCAAAT CGCTTTTGAT AATGGCTATT
  HP-1174  GAGCGCGATC AAGCCTTTTG TGTATCAAAT CGCTTTTGAT AATGGCTATT
  HP-1144  GAGCGCGATC AAGCCTTTTG TGTATCAAAT CGCTTTTGAT AATGGCTATT
  HP-1028  GAGCGCGATA AAGCCTTTTC TGTATCAAAT CGCTTTTGAT AATGGCTATT
   HP-870  GAGCGCAATA AAGCCTTTTG TGTATCAAAT CGCTTTTGAT AATGGCTATT
Consensus  GAGCGCgATa AAGCCTTTTg TGTATCAaAT CGCTTTTGAT AATGGCTATT

           1151                                                1200
    26695  CCACGACTTC TAAAATCCCT GATACCGCGC GAAACTTTGA AAATGGCAAT
      HPC  CCACCACTTC CAAAATCCCT GATACCGCGC GAAACTTTGA AAATGGCAAT
  HP-1163  CCACCACTTC CAAAATCCCT GATGCCGCGC GAAACTTTGA AAATGGCAAT
  HP-1174  CCACGACTTC CAAAATCCCT GATACCGCAC GAAACTTTGA AAATGGCAAT
  HP-1144  CCACGACTTC CAAAATCCCT GATACCGCAC GAAACTTTGA AAATGGCAAT
  HP-1028  CCACCACTTC TAAAATCCCT GATACCGCGC GAAACTTTGA AAATGGC---
   HP-870  CCACCACTTC TAAAATCCCT GATACCGCGC GAAATTTTGA AAATGGCAAT
Consensus  CCACcACTTC tAAAATCCCT GATACCGCgC GAAAcTTTGA AAATGGCaat

           1201                                                1250
    26695  TATAGTAAAA ACAGTGAACA AAACCACGCA TGGCACCCCA GCAATTATTC
      HPC  TATAGTAAAA ACAGCGAACA AAACCACGCA TGGCACCCCA GCAATTATTC
  HP-1163  TATAGTAAAA ACAGCGTACA AAGCCACGCA TGGCACCCCA GCAATTATTC
  HP-1174  TATAGTAAAA ACAGCGAACA AAACCACGCA TGGCACCCCA GCAATTATTC
  HP-1144  TATAGTAAAA ACAGCGAACA AAACCACGCA TGGCACCCCA GCAATTATTC
  HP-1028  TATAGTAAAA ACAGCGAACA AAACCACGCA TGGCACCCTA GAAATTATTC
   HP-870  TATAGTAAAA ACAGCGAACA AAACCACGCA TGGCACCCTA GCAATTATTC
Consensus  TATAGTAAAA ACAGcGAACA AAACCACGCA TGGCACCCcA GcAATTATTC
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           1251                                                 1300
    26695  TCGCAAGTTT TTAGGGCTTG TAACCTTGCA AGAAGCCTTG AGCCATTCGT
      HPC  TCGCAAGTTT TTAGGGCTTG TAACCTTGCA AGAAGCCTTA AGCCATTCGT
  HP-1163  TCGCAAGTTT TTAGGGCTTG TAACCTTGCA AGAAGCCTTA AGCCTTTCGT
  HP-1174  TCGCAAGTTT TTAGGGCTTG TAACCTTGCA AGAAGCTTTG AGCCATTCGT
  HP-1144  TCGCAAGTTT TTAGGGCTTG TAACCTTGCA AGAAGCTTTG AGCCATTCGT
  HP-1028  TCGCAAGTTT TTAGGGCTTG TAACCTTGCA AGAAGCTTTG AGCCATTCGT
   HP-870  TCGCAAGTTT TTAGGGCTTG TAACCTTGCA AGAAGCTTTG AGCCATTCGT
Consensus  TCGCAAGTTT TTAGGGCTTG TAACCTTGCA AGAAGCtTTg AGCCATTCGT

           1301                                                 1350
    26695  TAAATCTAGC CACGATCAAT TTAAGCGATC AGCTTGGCTT TGAAAAAATT
      HPC  TAAATCTAGC CACGATCAAT TTAAGCGATC AGCTTGGCTT TGAAAAAATT
  HP-1163  TAAATCTAGC CACGATCAAT TTAAGTGATC AGCTTGGCTT TGAAAAAATT
  HP-1174  TAAATCTAGC CACGATCAAT TTAAGCGATC AGCTTGGCTT TGAAAAAATT
  HP-1144  TAAATCTAGC CACGATCAAT TTAAGCGATC AGCTTGGCTT TGAAAAAATT
  HP-1028  TAAATCTAGC CACGATCAAT TTAAGCGATC AGCTTGGCTT TGAAAAAATT
   HP-870  TAAATCTGGC CACGATCAAT TTAAGCGATC AGCTTGGCTT TGAAAAAATT
Consensus  TAAATCTaGC CACGATCAAT TTAAGCGATC AGCTTGGCTT TGAAAAAATT

           1351                                                 1400
    26695  TATCAATCTT TAAGCGATAT GGGGTTTAAA AACCTCCCTA AGGACTTGTC
      HPC  TATCAATCTT TAAGCGATAT GGGGTTTAAA AACCTCCCTA AGGACTTGTC
  HP-1163  TATCAATCTC TAAGCGATAT GGGGTTTAAA AACCTCCCTA AGGACTTGTC
  HP-1174  TATCAATCTT TAAGCGATAT GGGGTTTAAA AACCTCCCTA AAGACTTGTC
  HP-1144  TATCAATCTT TAAGCGATAT GGGGTTTAAA AACCTCCCTA AAGACTTGTC
  HP-1028  TATCAATCTT TAAGCGATAT GGGGTTTAAA AACCTCCCTA AAGACTTGTC
   HP-870  TATCAATCTT TAAGCGATAT GGGGTTTAAA AATCTCCCTA AAGACTTGTC
Consensus  TATCAATCTT TAAGCGATAT GGGGTTTAAA AAcCTCCCTA AaGACTTGTC

           1401                                                 1450
    26695  TATTGTGTTA GGGAGCTTTG CTATCTCACC CATTGATGCA GCTGAAAAGT
      HPC  TATCGTGTTA GGGAGCTTTG CTATCTCACC CATTGATGCG GCTGAAAAGT
  HP-1163  TATCGTGTTA GGGAGCTTTG CTATCTCACC CATTGATGCG GCTGAAAAGT
  HP-1174  TATTGTATTA GGGAGCTTTG CTATCTCACC GATTGAAGCG GCTGAAAAGT
  HP-1144  TATTGTATTA GGGAGCTTTG CTATCTCACC GATTGAAGCG GCTGAAAAGT
  HP-1028  TATTGTGTTA GGGAGCTTTG CTATCTCACC GATTGAAGCG GCTGAAAAGT
   HP-870  TATTGTGTTA GGAAGCTTTG CTATCTCACC GATTGAAGCG GCTGAAAAAT
Consensus  TATtGTgTTA GGgAGCTTTG CTATCTCACC gATTGAaGCg GCTGAAAAgT

           1451                                                 1500
    26695  ATTCTTTATT TTCTAATTAC GGCACCATGC TCAAACCCAT GCTCATTGAA
      HPC  ATTCTTTATT TTCTAATTAC GGCACCATGC TCAAACCCAT GCTCATTGAA
  HP-1163  ATTCTTTATT TTCTAATTAC GGCACCATGC TCAAACCCAT GCTCATTGAA
  HP-1174  ATTCTTTATT TTCTAATTAC GGCACCATGC TCAAACCCAT GCTCATTGAA
  HP-1144  ATTCTTTATT TTCTAATTAC GGCACCATGC TCAAACCCAT GCTCATTGAA
  HP-1028  ATTCTTTATT TTCTAATTAC GGCACCATGC TCAAACCCAT GCTCATTGAA
   HP-870  ATTCTTTATT TTCTAATTAC GGCACCATGC TCAAACCCAT GCTCATTGAA
Consensus  ATTCTTTATT TTCTAATTAC GGCACCATGC TCAAACCCAT GCTCATTGAA
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           1501                                                1550
    26695  AGCATCACTA ACCAACAAAA CGATGTCAAA ACTTTCACGC CTATGGAAAC
      HPC  AGCATCACTA ACCAACAAAA CGATGTCAAA ACTTTCACGC CTATGGAAAC
  HP-1163  AGCATCACTA ACCAACAAAA CGATGTCAAA ACTTTCACGC CTATGGAAAC
  HP-1174  AGCATTACCG ATCAGCAAAA CAATATCAAA ACTTTCACGC CCATTGAAAC
  HP-1144  AGCATTACCG ATCAGCAAAA CAATATCAAA ACTTTCACGC CCATTGAAAC
  HP-1028  AGCATCACTG ACCAACAAAA CGATGTCAAA ACTTTCACGC CCATTGAAAC
   HP-870  AGCATCACTG ACCAACAAAA CGATGTCAAA ACTTTCACGC CCATGGAAAC
Consensus  AGCATcACtg AcCAaCAAAA CgATgTCAAA ACTTTCACGC CcATgGAAAC

           1551                                                1600
    26695  CAAAAAGATC ACCTCCAAAG AACAGGCTTT TTTAACCCTT TCAGTGCTGA
      HPC  CAAAAAGATC ACCTCCAAAG AACAGGCTTT TTTAACCCTT TCAGTGCTGA
  HP-1163  CAAAAAGATC ACCTCCAAAG AACAGGCTTT TTTAACCCTT TCAGTGCTGA
  HP-1174  CAAAAAGATC ACCTCCAAAG AACAGGCTTT TTTAACCCTT TCAGTGCTGA
  HP-1144  CAAAAAGATC ACCTCCAAAG AACAGGCTTT TTTAACCCTT TCAGTGCTGA
  HP-1028  CAAAAAGATC ACCTCCAAAG AACAGGCTTT TTTAACCCTT TCAGTGCTGA
   HP-870  CAAAAAGATC ACCTCTAAAG AGCAAGCTTT TTTAACCCTT TCAGTGCTGA
Consensus  CAAAAAGATC ACCTCcAAAG AaCAgGCTTT TTTAACCCTT TCAGTGCTGA
           1601                                                1650
    26695  TGGATGCGGT AGAAAATGGC ACAGGGAGTT TGGCTCGCAT TAAAGGTTTA
      HPC  TAAATGCGGT AGAAAACGGC ACAGGGCGTT TGGCTCGCAC TAAAGGTTTA
  HP-1163  TAAATGCGGT AGAAAACGGC ACAGGGCGTT TGGCTCGCAC TAAAGGTTTA
  HP-1174  TGAATGCGGT AGAAAACGGC ACAGGAAATT TGGCTCGCAT TAAAGGTTTA
  HP-1144  TGAATGCGGT AGAAAACGGC ACAGGAAATT TGGCTCGCAT TAAAGGTTTA
  HP-1028  TGAATGCGGT AGAAAATGGC ACGGGGAGTT TGGCGCGTAT TAAAGGTTTA
   HP-870  TGAATGCGGT AGAAAATGGC ACAGGGAGTT TGGCTCGCAT TAAAGGTTTA
Consensus  TgaATGCGGT AGAAAAtGGC ACaGGgagTT TGGCtCGcAt TAAAGGTTTA

           1651                                                1700
    26695  GAAATCGCCG GTAAAACCGG GACTTCTAAC AACAATATTG ACGCTTGGTT
      HPC  GAAATCGCCG GTAAAACCGG GACTTCTAAC AACAATATTG ACGCTTGGTT
  HP-1163  GAAATCGCCG GTAAAACCGG GACTTCTAAC AACAATATTG ACGCTTGGTT
  HP-1174  GAAATTGCCG GTAAAACCGG GACTTCTAAC AATAATATTG ATGCTTGGTT
  HP-1144  GAAATTGCCG GTAAAACCGG GACTTCTAAC AATAATATTG ATGCTTGGTT
  HP-1028  GAAATCGCCG GTAAAAGCGG GACTTCTAAC AACAATATTG ATGCTTGGTT
   HP-870  GAAATTGCCG GTAAAACCGG GACTTCTAAC AACAATATTG ATGCTTGGTT
Consensus  GAAATcGCCG GTAAAAcCGG GACTTCTAAC AAcAATATTG AtGCTTGGTT

           1701                                                1750
    26695  CATTGGCTTT ACCCCCACCT TGCAAAGCGT GATCTGGTTT GGGAGGGACG
      HPC  CATTGGCTTT ACCCCCACCT TGCAAAGCGT GATCTGGTTT GGGAGAGACG
  HP-1163  CATTGGCTTT ACCCCCACCT TGCAAAGCGT GATCTGGTTT GGGAGAGACG
  HP-1174  CATTGGTTTT ACCCCCACCT TACAAAGCGT GATCTGGTTT GGGAGAGACG
  HP-1144  CATTGGTTTT ACCCCCACCT TACAAAGCGT GATCTGGTTT GGGAGAGACG
  HP-1028  CATTGGCTTT ACCCCCACCT TACAGAGCGT GATTTGGTTT GGGAGGGACG
   HP-870  CATTGGCTTT ACCCCCACCT TACAAAGCGT GATCTGGTTT GGGAGAGACG
Consensus  CATTGGcTTT ACCCCCACCT TaCAaAGCGT GATcTGGTTT GGGAGaGACG
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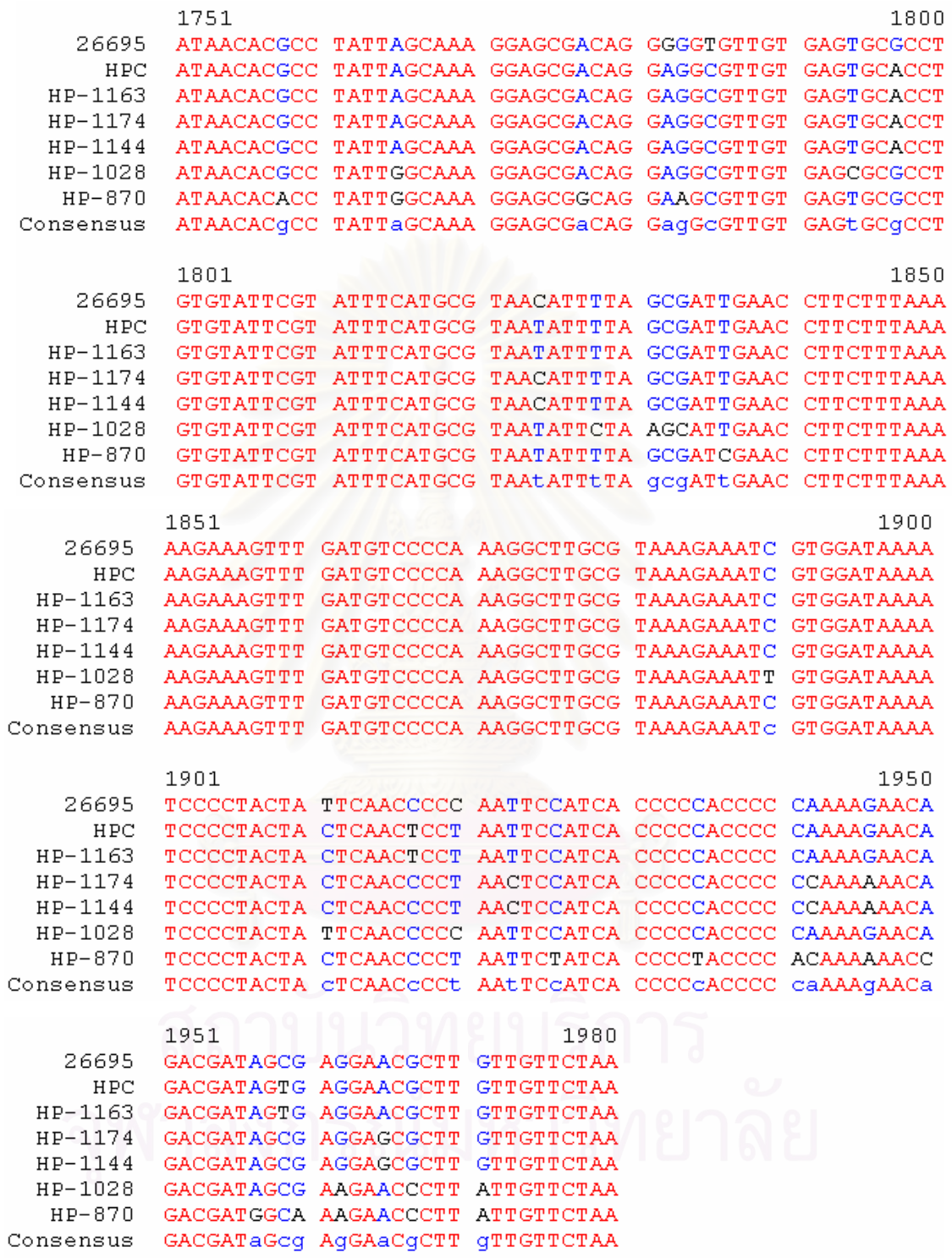

Abbreviations: A, adenine; C, cytosine; G, guanine; T, thymine; 26695, *H. pylori* 26695; HP, *H. pylori*; HPC, *H. pylori* NCTC 11637

1. 64 codon on DNA

First Position	Second Position				Third Position
	T	**C**	A	**G**	
T	**F**	**S**	**Y**	**C**	**T**
	F	**S**	**Y**	**C**	**C**
	L	**S**	**STOP CODON**	**STOP CODON**	**A**
	L	**S**	STOP CODON	**W**	**G**
C	**L**	**P**	**H**	**R**	**T**
	L	**P**	**H**	**R**	**C**
	L	**P**	**Q**	**R**	**A**
	L	**P**	**Q**	**R**	**G**
A	**I**	**T**	**N**	**S**	**T**
	I	**T**	**N**	**S**	**C**
	I	**T**	**K**	**R**	**A**
	M	**T**	**K**	**R**	**G**
G	**V**	**A**	**D**	**G**	**T**
	V	**A**	**D**	**G**	**C**
	V	**A**	**E**	**G**	**A**
	V	**A**	**E**	**G**	**G**

Abbreviation: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, trytophan; Y, tyrosine

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

BIOGRAPHY

Miss. Punjapon Prasurthsin was born on November 4, 1971 in Ubonrajathanee, Thailand. She graduated with the Bachelor degree of Agronomy from the Faculty of Agriculture, Ubon Rajathanee University in 1994. She is currently a member of Bacteriology Unit, Department of Microbiology, King Chulalongkorn Memorial Hospital and was given the opportunity to pursue her MS degree in the Inter-Department of Medical Microbiology, Faculty of Graduate School, Chulalongkorn University since 2002.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย