การตรวจหาการดื้อยา Clarithromycin ของเชื้อ *Helicobacter pylori* ที่แยกได้จากผู้ป่วยไทย

นางสาวสมวัย ลีตรานนท์

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DETECTION OF CLARITHROMYCIN RESISTANCE IN *HELICOBACTER PYLORI* ISOLATED FROM THAI PATIENTS

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สมวัย ลีตรานนท์ : การตรวจหาการดื้อยา Clarithromycin ของเชื้อ *Helicobacter pylori* ที่แยกได้จากผู้ป่วยไทย (DETECTION OF CLARITHROMYCIN RESISTANCE IN *HELICOBACTER PYLORI* ISOLATED FROM THAI PATIENTS) อาจารย์ที่ปรึกษา : รศ.ดร. สมหญิง ธัมวาสร, 100 หน้า, ISBN 974-17-2730-5

Clarithromycin เป็นยาที่ใช้ในการกำจัดเชื้อ *Helicobacter pylori* และการดื้อต่อยานี้มักเป็นสาเหตุหลักของการรักษาที่ล้มเหลว เพื่อตรวจหาความชุกของการดื้อยา clarithromycin ของเชื้อ *H.pylori* ที่แยกได้จากผู้ป่วย ได้ใช้วิธี Epsilometer test และ Agar dilution ทดสอบหาความไวรับของยา clarithromycin วิเคราะห์การกลายพันธุ์ในส่วน 23S rRNA gene ที่มีความสัมพันธ์กับการดื้อยาในกลุ่ม macrolide โดยการเพิ่มปริมาณ DNA ด้วยเทคนิค PCR และ reverse dot blot hybridization การศึกษาในครั้งนี้ ได้ทำการเพาะเชื้อจากชิ้นเนื้อกระเพาะอาหารจำนวน 150 ตัวอย่าง พบว่าตรวจแยกเชื้อ *Helicobacter pylori* ได้จาก 71 (47.33%) ตัวอย่าง เชื้อที่แยกได้ 71 สายพันธุ์ ให้ผลดื้อต่อยา clarithromycin จำนวน 31 (43.66%) สายพันธุ์ และไม่ดื้อต่อยา clarithromycin จำนวน 40 (56.34%) สายพันธุ์ เชื้อทั้งหมด 31 สายพันธุ์ที่มีการดื้อต่อยา clarithromycin เกิดการกลายพันธุ์ที่ส่วน 23S rRNA gene ส่วน 40 สายพันธุ์ที่ไม่ดื้อต่อยา clarithromycin จะไม่เกิดการกลายพันธุ์ที่ส่วน 23S rRNA gene จากการวิเคราะห์ลำดับเบสในส่วน 23S rRNA gene ไม่พบการกลายพันธุ์ที่ตำแหน่งที่เคยมีรายงานมาก่อน เช่น A2142G ในทางตรงกันข้ามพบว่าเชื้อทุกสายพันธุ์ที่ดื้อต่อยา clarithromycin เกิดการกลายพันธุ์ที่ตำแหน่ง T2182C พบเชื้อ 2 สายพันธุ์มีการกลายพันธุ์ที่ 2 ตำแหน่ง คือ T2182C และ A2143G และ 3 สายพันธุ์ เกิดการกลายพันธุ์ที่ 2 ตำแหน่ง คือ T2182C และ A2223G การศึกษาในครั้งนี้พบว่าวิธี reverse dot blot hybridization ให้ผลตรงกับวิธีวิเคราะห์หาลำดับเบส

สาขาวิชา จุลชีววิทยาทางการแพทย์ (สหสาขาวิชา) ลายมือชื่อนิสิต.....

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Clarithromycin is a drug of choice to eradicate *H.pylori* and resistance to this drug is a predominant cause of treatment failure. In order to determine the prevalence of clarithromycin resistance in *H.pylori* isolated from Thai patients, drug susceptibility test was determined by the epsilometer test and agar dilution. Mutations in the 23S rRNA gene that are associated with macrolide resistance were analyzed by PCR and reverse dot blot hybridization. Gastric biopsy specimens from 150 patients were cultured for *H.pylori* isolation. Seventy-one (47.33%)gastric biopsy specimens yielded *H.pylori*-positive results. Of the 71 isolates determined by MIC testing, 31 (43.66%) were resistant and 40 (56.34%) were susceptible to clarithromycin. All of the 31 resistant isolates contained 23S rRNA gene mutations whereas 40 susceptible isolates contained wild-type sequences. Sequence analysis of the 425-bp PCR product (portion of the 23S rRNA gene) did not reveal mutation such as that described at position A2142G. On the contrary, our findings demonstrated a T to C transition at position 2182 for all resistant isolates. Two isolates contained double mutations at position T2182C and A2143G and 3 isolates contained double mutations at positions T2182C and A2223G. Reverse dot blot hybridization gave concordant results for mutation detection as those obtained from sequencing.

Field of study : Medical Microbiology Student's signature……...

Academic year 2002 Advisor's signature……..

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ABBREVIATIONS

A	adenosine
bp	base pair
C	cytosine
CO_2	carbon dioxide
°C	degree celsius
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
ddATP	dideoxyadenosine 5'-triphosphate
ddCTP	dideoxycytidine 5'-triphosphate
ddGTP	dideoxyguanosine 5'-triphosphate
ddTTP	dideoxythymidine 5'-triphosphate
DDW	double distilled water
ddNTPs	dideoxynucleotide-tri-phosphate
dGTP	deoxyguanosine 5'-triphosphate
DNA	deoxynucleic acid
dNTPs	deoxynucleotide-tri-phosphate
dTTP	deoxythymidine 5'-triphosphate
DW	distilled water
EDAC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA	ethyenediamine tetraacetic acid
et al.	et alii
E-test	epsilometer test
g	gram
G	guanosine
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
hr	hour
i.e.	id est
KCl	potassium chloride

M	molar
mg	milligram
$MgCl_2$	magnesium chloride
MIC	minimal inhibitory concentration
min	minute(s)
ml	millilitre
mM	millimolar
mmol	millimolar
NaCl	sodium chloride
$NaHCO_3$	sodium hydrogen carbonate
Na_2HPO_4	sodium phosphate dibasic, anhydrous
NaOH	sodium hydroxide
PCR	polymerase chain reaction
pmol	picomol
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
23S rRNA	twenty three subunit ribosomal ribonucleic acid
SDS	sodium dodecyl sulfonate
sec	second
T	thymidine
TAE	Tris-acetate-EDTA
Taq	Thermus aquaticus
T_d	dissociate temperature
T_m	melting temperature
Tris	Tris-(Hydroxymethyl)-aminoethane
U	unit
μg	microgram
μl	microliter
μM	micromolar
UV	ultraviolet
V	volt

WHO	World Health Organization
w/v	weight per volume

CHAPTER I

INTRODUCTION

Helicobacter pylori is a microaerophilic gram-negative spiral bacterium that infects the human gastric mucosa (1). *H.pylori* infects more than half of the world population. Persistent infection with *H.pylori* is associated with chronic gastric and peptic ulcer disease and may eventually result in the development of atrophic gastritis, mucosa-associated lymphoid tissue lymphoma, and gastric cancer (2, 3, 4, 5, 6, 7).

According to the U.S. National Institutes of Health Consensus Development Conference was recommended the addition of antimicrobial agents to antisecretory drugs were recommended for the treatment of patients with *H.pylori* associated peptic ulcer disease (8). Infection with *H.pylori* can be effectively treated by a combination of a proton pump inhibitor and/or an H_2- receptor antagonist with multiple antibiotics. Metronidazole, amoxicillin, clarithromycin and tetracycline are frequently included in triple or quadruple regiments (9, 10). The antimicrobial agents that are most often used are therapy with two of these antibiotics plus on antisecretory drug. The treeatment usually achieves an eradication rate above 80% (11, 12, 13, 14, 15). Resistance to antimicrobial agents has a significant impact on the efficacy of anti-*Helicobacter* treatment. Virtually all *H.pylori* strains are susceptible to amoxicillin, while 5 to 15% of strains are resistant to clarithromycin and 10 to 50% of strains are resistant to metronidazole (16, 17, 18, 19, 20, 21). Resistance against clarithromycin and metronidazole is also frequently associated and is of particular clinical importance as these drugs are used in almost all standard *H.pylori* eradication regimens (22, 23). Particularly, the development of clarithromycin resistance among *H.pylori* strains, has become a predominant cause of failure of therapy including this drug (24, 25, 26). Resistance to metronidazole is observed in 10 to 50% of the cases in developed countries and can be as high as 90% in developing countries (27). The prevalence of macrolide-resistant strains varies among countries and ranges from less than 2% in the Netherlands and Norways (20, 28) to more than 10% in France and some other countries (27, 29, 30, 31). Moreover, the prevalence of resistant strains appears to be

increasing (32, 33, 34). Resistance to clarithromycin decreases the effectiveness of antibiotic therapy by an average of 55% (35, 36).

Clarithromycin is a component of the widely used regimens. However, macrolide resistance in *H.pylori* has been shown to occur at different rate (5 to 15%) in different countries (37) and is an important cause of the failure of these regimens. Moreover, *H.pylori* mutants resistant to macrolides are easily obtained by in vitro selection (38). Macrolides inhibit protein synthesis by stimulating dissociation of peptidyl-tRNA from the ribosome during the elongation reaction (39).

The major cause of macrolide resistance in *H.pylori* is due to several mechanisms including lack of macrolide binding to the ribosome target (23S rRNA components) and, to a lesser extent, macrolide inactivation by enzymes, impermeability of the bacterial membrane, and active drug efflux (43). The target modification usually involves a posttranscriptional modification (by methylation or point mutations) or a mutation situated in the peptidyltransferase domain (domain V) of the 23S rRNA (44), while mutations of the ribosomal proteins have also been described (43). This mutation (A to G at positions 2142 or 2143, formerly labeled 2058 and 2059, respectively) was found for the first time by Versalovic et al. in *H.pylori* strains isolated in the United States (45, 46).

Since macrolide resistance is clinically important, and because the prevalence of resistant strains is increasing, there is a clear need for rapid and accurate diagnostic methods to determine macrolide resistance. The conventional method to determine the antibiotic resistance of *H.pylori* is based on the analysis of cultured strain by agar dilution or epsilometer (E-test) (47). These methods are tedious and strongly dependent on the experimental conditions and subjective interpretation and hence are not always reproducible. DNA-based diagnostic methods may offer a rapid and reliable alternative approach for macrolide susceptibility testing. Furthermore, molecular assays can be applied directly on gastric biopsy specimens, without the need for in vitro culture of the bacterium (48). Therefore, we have developed a PCR-based reverse hybridization assay that permits simultaneous identification of the different mutations in the 23S rRNA.

CHAPTER II

OBJECTIVE

1. Determine the rate of clarithromycin resistance in *Helicobacter pylori* from Thai patients

2. Determine the association of point mutations in the 23S rRNA gene with clarithromycin resistance in *Helicobacter pylori*

3. Develop rapid molecular method for detection of point mutations associated with resistance of *Helicobacter pylori* to clarithromycin by reverse hybridization

CHAPTER III

LITERATURE REVIEW

1. Historical perspective

Helicobacter pylori was first isolated in 1982 by Marshall and Warren in western Australia from gastric mucus of patients with chronic gastritis and duodenal ulcers. These investigators referred to the microorganism as Campylobacter-like bacteria (1, 49). Skirrow, a microbiologist in the Campylobacter field, suggested that if the organisms were proved to be a member of the genus *Campylobacter*, the name *Campylobacter pyloridis* would be apt because of their specific location and association (50). Successful culture of the bacterium resulted in the acceptance of this name, which was later corrected to *Campylobacter pylori* (51, 52).

Yet almost from its initial cultivation it was suspected that perhaps *Campylobacter pylori* was not a true *Campylobacter*. Early electron micrographs showed multiple sheathed flagella at one pole of the bacterium, in contrast to the single bipolar unsheathed flagellum typical of *Campylobacter* species (53). Further studies revealed that the organism differed sufficiently from true campylobacters to justify the formation of a new genus *Helicobacter* (54). It soon becomes apparent that similar organisms colonized the stomach of a wide variety of animals other than humans, and that certain spiral bacteria colonizing the intestines of rodents and other animals also belonged to *Helicobacter*. Ongoing study of this organism, particularly at the genetic level, resulted in the formation of a new genus, *Helicobacter* (helico = curved, bacter = staff), with *Helicobacter pylori* as the type species (55), the first member of the new genus *Helicobacter* (56).

The analysis of the sequence of the 16S rRNA gene led to the differentiation of *Helicobacter pylori* from *Campylobacter* species and this molecular tool also has been instrumental in the classification of the other members of this genus. Other important features that differentiate *Helicobacter* species from *Campylobacter* species included the

possession of sheathed flagella, unique fatty acid profile, lack of respiratory quinones, active urease enzyme, and a distinct protein profile.

2. General characteristics of the genus *Helicobacter*

2.1 Cellular morphology and ultrastructure

Helicobacter are non-spore-forming gram-negative bacteria. The cellular morphology may be curved, spiral, or fusiform, typically 0.2 to 1.2 μm in diameter and 1.5 to 10.0 μm long and have rounded ends. The spiral wavelength may vary with the age, the growth conditions and the species identity of the cells. In old cultures or those exposed to air, cells may become coccoid.

Periplasmic fibers or an electron-dense glycocalyx or capsule-like layer has been observed on the cellular surface of several species (57,58,59,60). Electron-dense granular bodies have been observed in *H.pylori* (61) and *H. rodentium* (60). In *H.pylori* these bodies are known to be aggregates of polyphosphate and may serve as a reserved energy source.

Helicobacter cells are motile with a rapid cork-screw-like or slower wave-like motion due to flagella activity. Strains of most species have bundles of multiple sheathed flagella with a polar or bipolar distribution.

The genus *Helicobacter* at present consists of 18 offical genera with another 10 potentially novel species (62). Of the offical genera, 8 are of gastric origin and the remaining 10 are found in the intestinal tract of a wide variety of animal species in Table 1. *H.pylori* and *H.heilmanii* are the only 2 species which have been associated with human gastric disease. *H.cinaedi* and *H.fennelliae* are causes of enteritis and proctolitis, especially in homosexual men, and they may sometimes also cause bacteremia. Distinguished features of 4 important members of the *Helicobacter* genus are compared in Table 2.

Table 1. Natural hosts and usual site of isolation of *Helicobacter* species

***Helicobacter* species**	**Main Host**	**Origin**
H.pylori	Human	Gastric
H.mustelae	Ferret	Gastric
H.nemestrinae	Macaque monkey	Gastric
H.felis	cat,dog	Gastric
H.acinomychis	Cheetah	Gastric
H.bizzozeronii	Dog	Gastric
H.salmonis	Dog	Gastric
H.heilmannii	Human, cat, dog, pig	Gastric
H.cinaedi	Human, hamster	Intestinal
H.fennelliae	Human, hamster	Intestinal
H. muridarum	Rat, mouse	Intestinal
H.canis	Dog	Intestinal
H.pullorum	Poultry	Intestinal
H.pametensis	Tern	Intestinal
H.hepaticus	Mouse	Intestinal
H.bilis	Mouse	Intestinal
H.cholecystus	Hamster	Intestinal
H.rodentium	Mouse	Intestinal

Table 2. Important members of the genus *Helicobacter*.

Name	Host	Features	Disease associations and comments
H.pylori	Human	multiple (4-6) sheathed flagella at one end	Causes gastritis in human. also found sometimes in domesticated or caged animals, e.g. monkeys, pigs, cats.
H.heilmannnii	Human, cat, dog	Corkscrew appearance with between a and 20 turns, at least 12 sheathed flagella at one end, no axial periplasmic fibers noted.	about 1% of human gastritis cases are caused by this bacterium, presumably acquire from cats and dogs.
H.mustelae	Ferret	Several randomly placed flagella	Gastritis and ulcerations commonly develop in ferrets, useful model for studying pathogenic mechanisms.
H.felis	cat,dog	Differs only from *H.heilmannnii* by the presence of axial periplasmic fibers noted by electron microscopy	Isolated from cats, can be propagated in mice, useful in screening trials for anti-*H.pylori* chemotherapy agent.

2.2 Growth characteristic

In laboratory conditions, strains typically grow under strictly microaerobic condition at 37°C. No growth is observed in aerobic conditions. Helicobacters will grow at 37°C on a variety of rich agar bases supplemented with 5% whole blood or serum. Many species require fresh media with moist agar surfaces for optimal growth conditions, though this is not usually the case for *H.pylori*.

3. *Helicobacter pylori*

3.1 Morphology

Helicobacter pylori is a spiral to curved, rod-shaped bacterium approximately 0.5 μm in diameter and 3 to 5 μm long. This organism possesses the characteristic ultrastructure of a gram-negative bacterium. In older cultures, cells are seen to ball up, form U-shaped structures and lose their cytoplasmic cylinders and membrane integrity, resulting in the formation of coccoid cells (63). It has been proposed that this coccoid form is a viable but nonculturable form of the organism, which allows it to survive in hostile environments outside the gastric mucus (64).

Helicobacter pylori has 4 to 7 polar sheathed flagella which enable the bacterium to move freely in viscous environments as gastric mucus (65). Several studies have reviewed that this motility is essential for the bacterial colonization of its host (66). The flagella sheath is a membrane containing proteins and lipopolysaccharides which probably protects the flagella filaments from the gastric acidity (67). The flagella filament contains two different flagellin proteins, Fla A and Fla B, both of which have been shown to be necessary for the motility of the organism.

3.2 Microbiological detection of *Helicobacter pylori*

3.2.1 The atmosphere for culture of *Helicobacter pylori*

In general, primary cultures of *H.pylori* have less oxygen tolerance than most *Campylobacter* species with a growth maximum at 3 to 7% of O_2. *H.pylori* is usually grown in jars with gas-generation kits (68, 69, 70) or a standard microaerobic atmosphere

in CO_2 incubation or anaerobic chambers with a microaerobic atmosphere. Most studies with standardized atmosphere for culture of *H.pylori* have used 2 to 5% O_2, 5 to 10% (optimal closer to 10%) CO_2, and 0 to 10% H_2; high humidity are required for growth (71, 72, 73, 74, 75, 76, 77, 78). Optimal growth is obtained at 37°C after 4 to 5 days for primary culture or 2 days for subsequent subculture.

Plates must be incubated in a microaerobic environment (5 to 7% O_2) incorporating increased CO_2 (5 to 10%) and a high relative humidity. This environment may be created by using the campy Pak system (BBL Microbiology System, Cockeysville, Md.) in an anaerobic jar with a moistened towel in the bottom. A single evacuation of an anaerobic jar to 220 mm Hg (1 mm Hg = 133.322 Pa) and replacement with an anaerobic gas mixture (10% CO_2, 10% H_2, and 80% N_2) yield an acceptable atmosphere of 5% O_2, 7% CO_2, 8% H_2 and 80% N_2 (79). The Campy Pouch (BBL), which holds one or two plates, has also been effective for subculture. Because an acceptable atmosphere is generated in the pouch, primary isolation is predicted even though no formal evaluation has been published. All biochemical and susceptibility tests for these organisms require microaerobic conditions (80).

3.2.2 Nonselective and selective media for growing *H.pylori*

H.pylori can grow on different solid media containing blood or blood products (blood or lysed blood agar plates). Most studies have Brucella agar or Columbia agar as the agar base. An amount of 7 to 10% blood improves the growth of *H.pylori* as compared with 5% blood. Horse blood may also improve the growth of *H.pylori* as compared to sheep blood (81, 82). Supplement of agar with cyclodextrin B can be used for blood-free culture media for *H.pylori* but with large differences between different batches of cyclodextrin (83). Egg yolk emulsion agar has also been described as a blood-free medium for growth of *H.pylori* (84).

Often *H.pylori* grows poorly or not at all on selective media containing antibiotics. Skirrows and Dents selective media seen to be the best available commercial selective media and have been used in several studies (85, 86, 87, 88). There seem to be greater differences between horse and sheep blood agar, in with and without antibiotics (81, 82). By comparing agar plates containing 5% horse blood, 10% horse blood, 7%

lysed horse blood, 7% lysed horse blood with trimetroprim and selective campylobacter plates, revealed that *H.pylori* with more plates than on the other media, but the numbers of *H.pylori*-positive patients were almost equal with all media.

Like Campylobacters, *H.pylori* is strictly microaerophilic and CO_2 (5 to 10%) and high humidity are required for growth. *H.pylori* requires media containing supplements similar to those used for Campylobacters ; blood, serum, haemin, starch, or charcoal. However, *H.pylori* is inhibited by the bisulphate in the FBP Campylobacter "aerotolerance supplement". Growth is best on media such as moist freshly prepared heated (chocolate) blood agar, and nutrient-rich media such as brain heart infusion agar or brucella agar supplement with 5 to 7% horse blood are adequate as nonselective media (89). An egg yolk emulsion agar with Columbia agar base has also been shown to yield excellent nonselective growth of *H.pylori* when compared to other culture media for *H.pylori* as shown in Table 3.(90).

Usually *H.pylori* grows slowly in liquid media with formation of a high numbers of coccoid forms (84, 91). Contaminating microorganisms (Staphylococci, yeasts, etc.) usually grow much faster than *H.pylori* and make liquid media useless for primary culture of biopsies. Becauses of the risk of contaminated samples, a selective medium is usually recommended in addition to the nonselective media for routine culture.

3.2.3 Transportation and handling of biopsies for *H.pylori*

Gastric biopsy specimens are the only ones likely to be used for the primary isolation of *H.pylori*. they should be transported in a moist state and cultured within 2 hours of collection. Storage beyond this time should be at 4°C or at -20°C if the period is more than 2 days, whereas a higher temperature (about 20°C) decreases the number of positive cultures significantly (92, 93, 94). Thus, a decrease in culture rate of about 15% was found when biopsies were transported or stored overnight. A long transportation time decreases the number of *H.pylori* especially after antibiotics therapy, and if the number of bacteria is low, culture may be become false negative.

Various transport media have been described for transporting biopsy samples, including cysteine brucella broth, normal saline, glucose, milk, stuart's medium, semi-solid agar, brain-heart infusion broth and Clary-Blair medium (95).

Table 3. Comparison of culture media and atmospheric conditions for growth of *H.pylori* (90)

Agar Media	Conditions of Incubation				
	Anaerobe Jar (no Catalyst)	Campy GasPak Jar	Anaerobe Jar (Catalyst)	Poly Bag	Atmosphere Air with 5%CO_2
Brucella with 5%sheep blood	Best growth β-hemolysis	Very good growth β-hemolysis	No growth	No growth	No growth
Tryptic soy with 5%sheep blood	Very good growth	good growth	No growth	No growth	No growth
Brain heart infusion with 5% horse blood	Very good growth	good growth	No growth	No growth	No growth
Chocolate (BD)	No growth	No growth	No growth	No growth	No growth
Chocolate (GIBCO)	Small colonies	Small colonies	No growth	No growth	No growth
Chocolate (freshly)	Very small colonies	Very small colonies	No growth	No growth	No growth
Campy-BAP	No growth	No growth	No growth	No growth	No growth

3.2.4 Specimens for culture of *H.pylori*.

H.pylori is the microorganism most frequently found in the human gastric mucosa in association with gastric epithelial cells, but other curved bacteria have also been found in the gastric mucosa.

Gastric specimens**.** *H.pylori* is most regularly found in the antral part of human gastric mucosa of untreated persons. In persons treated with acid-suppressive drugs (proton pump inhibitors and H_2 antagonists), *H.pylori* may be present in higher numbers in the body of the stomach. *H.pylori* is more frequently found in gastric antrum than in duodenal biopsies even in persons with duodenitis and duodenal ulcer. *H.pylori* can only be cultured from gastric juice in about 15% of persons with *H.pylori* cultured from gastric antrum and from less than 50% of esophageal biopsies from untreated persons with esophagitis, even though *H.pylori* can be cultured from gastric antrum (71, 73). The number of biopsies necessary to diagnose *H.pylori* by culture has been estimated in a study where more than 95% of *H.pylori* was cultured from one antral biopsies (74).

Extragastric specimens. *H.pylori* has occasionally been cultured from ectopic gastric mucosa in Meckel's diverticulum, esophagus, rectum, urinary bladder, dental plaque, and fecae (75, 76, 77, 78, 79, 80, 81). Recently, *H.pylori* has also been detected by PCR in specimens from gallbladder and liver (82). No systematic studies have been carried out to recommend optimal sample sites for extragastric *H.pylori* infections. Culture-confirmed microbiological identification is preferable to ensure the bacteriological diagnosis of isolates from these sites, at least until molecular biological methods have been better evaluated than they are today.

3.2.5 Identification of *H.pylori*

The optimal temperature of incubation is 35 to 37°C. Colonies from primary isolation are generally observed by 3 to 4 days of incubation. Colonies of *H.pylori* are small (0.5 to 2 mm), translucent to yellowish colonies on 7% lysed horse blood agar and with translucent to pale grayish colonies of 0.5 to 1 mm in size on blood agar. In very young cultures, *H.pylori* may appear as almost straight rods on microscopy. After 3 to 5 days of incubation the bacteria look pleomorphic, with irregular curved rods, several

being U shaped. In old cultures, *H.pylori* appears as degenerative coccoid forms that Gram stain poorly. Because of their small size, *H.pylori* colonies may be difficult to identify and isolate when there are few colonies and additional contaminating oral microbiota is present. Some contaminating microorganisms may grow as small colonies but usually differ from *H.pylori* in color.

H.pylori is biochemically closely related to *Campylobacter*, *Arcobacter*, and *Wollinella* species but also resembles *Bacteroides*, *Thiovulum*, and *Selenomonas* species. They are all characterized as being gram-negative rods that are able to grow microaerobically or anaerobically. The rods may be more or less curved, depending on the growth conditions. The urease reaction is a key reaction in identifying *Helicobacter* species, but some *Campylobacter lari* strains are urease positive and at least one urease-negative *H.pylori* strain has been isolated from a patient. Several Helicobacter species are gram-negative, motile curved rods that are oxidase, catalase, and urease positive, and it may, therefore, be necessary to undertake protein profiles or genomic analysis to ensure the correct identification. See Table 4 for a summary of tests designed to identify *H.pylori*.

Table 4. Summary of tests for detection of *H.pylori*

Test	**Sensitivity (%)**	**Specificity (%)**	**Cost***	**Endoscopy required**	**Comments**
Culture	77-94	100	+++	Yes	"gold standard"
Histology	93-99	95-99	+++	Yes	Demonstrate host response and integrity of mucosa
Rapid urease test (CLO test)	86-97	86-98	+	Yes	Can be performed rapidly
^{13}C breath test (non radioactive)	96-100	80-99	++	No	Preferred for pregnant women and children; not widely available;excellent for early posttreatment tracking
^{14}C breath test	90-100	92-100	++	No	Small amount of radiation exposure; excellent for posttreatment tracking
Serology	83-98	56-100	+	No	Readily available; useful seroepidemiological tool; limited value in short-term tracking of therapy

* + = least expensive, ++ = moderately expensive, +++ = most expensive

4. Treatment of *H.pylori*

Because of the inactivity of certain antimicrobial agents in the acid environment of the stomach, most laboratory assays to predict antimicrobic activity against *H.pylori* do not predict *in vivo* results. *H.pylori* is susceptible to many antibiotics *in vivo*, although treatment *in vivo* is less trivival as the stomach is a difficult environment in which to carrying out successful antimicrobial therapy. Agar and broth dilution methods for susceptibility testing have been used (96, 97). A substantial decrease in efficacy is noted with triple-therapy regimens when metronidazole-resistants strains are detected, with success rates dropping from 90 to 40% with resistant organisms (98). *H.pylori* is susceptible to bismuth-containing compounds at levels achievable with oral dosing and is also sensitive to the proton pump inhibitors omeprazole and lansoprazole (99).

Infection with *H.pylori* can be effectively treated by a combination of proton pump inhibitors and/or H_2 receptor antagonists and antibiotics. Metronidazole, amoxicillin, clarithromycin, and tetracycline are frequently included in the triple or quadruple treatment regimens used to eradicate *H.pylori* (9, 100). Cure of *H.pylori* infection may reduce the risk of gastric adenocarcinoma and has been associated with regression of gastric mucosa-associated lymphoid tissue (MALT) lymphoma (5, 101, 102). The highest cure rates have been obtained with multiple antimicrobial treatment regimens that include an imidazole (i.e., metronidazole) or clarithromycin and that usually last 7 to 14 days (103, 104, 105).

Clarithromycin is a potent macrolide that has frequenctly been used in combination with other antimicrobial agents for the treatment of *H.pylori* infection (10, 105). However, the development of clarithromycin resistance among *H.pylori* strains has become a predominant cause of the failure of therapy incorporating clarithromycin (106, 107).

Much publicity is currently given to the widespread use of antibiotics and the threats posed by the emergence of pathogenic organism resistant to all available antibiotics. While it is generally accepted that treatment to eradicate *H.pylori* in patients with proved peptic ulcer is cost effective and benefits the patients and society, treatment to eradicate the organism in patients with non-ulcer dyspepsia (NUD) is a topic of

ongoing debate (108, 109). One reason used in the argument against the test and treat strategy is that empirical or indiscriminate antibiotic treatment of NUD could have an undesirable outcome in the emergence and persistence of resistant strains of *H.pylori* as well as the development of other resistant organisms in the gastrointestinal tract (110). Guide for treatment of *H.pylori* includes the macrolide clarithromycin, the 5-nitroimidazole metronidazole, as well as amoxicillin and tetracycline. Two of these antibiotics are used for *H.pylori* primary eradication in combination with a proton pump inhibitor in triple therapy regimens (112). Likewise, information on strain resistance following treatment failure is crucial in selecting an appropriate regimen as the development of bacterial resistance to antibiotics makes retreatment difficult (111, 112).

The clinical relevance of antibiotic resistance is also a controversial issue although its importance on outcome of therapy has been highlighted in several recent meta-analyses. For instance, van der Wouden and colleagues (113) addressed the influence of nitroimidazoles on the efficacy of treatment, based on world literature from 1993 to 1997, and calculated that eradication rates were 90% in metronidazole susceptible strains but <75% in resistant strains, although the choice of other drugs and treatment duration influenced the impact of resistance on treatment efficacy. A second meta-analysis based on studies published from 1983 to December 1997 to define the effect of pretreatment resistance to either metronidazole or clarithromycin on the success of therapy found that metronidazole resistance reduced effectiveness by an average of 37.7% but most striking was the finding that clarithromycin resistance reduced effectiveness by an average of 55% (35). In a recent German study, 86% of 554 isolates from patients in whom one or more eradication therapies had failed were resistant to both clarithromycin and metronidazole (114).

5. Clarithromycin resistance in *H.pylori*

Clarithromycin resistance of *H.pylori* is due to point mutation in the 23S rRNA (41, 42, 45, 115, 116, 117, 118). The major cause of macrolide resistance in *H.pylori* is the lack of binding of the macrolides to the 23S rRNA components of the bacterial ribosome due to a modification of the target site by methylation or point mutations in peptidyltransferase region of domain V of the 23S rRNA (119). *H.pylori* contains two

copies of the 23S ribosomal RNA gene, and at least five distinct points mutations have been reported that are associated with macrolide resistance. Versalovic et al. (45) found A to G transitions at two positions (A2142 to G and A2143 to G). Also, an A to C transversion (A2142 to C) was found to be related to resistance (41). Specifically, adenine-to-guanine transitions at either position 2058 or position 2059 (*Escherichia coli* coordinates) in the peptidyltransferase region of the 23S rRNA were in most cases associated with clarithromycin resistance. According to the new numbering scheme for *H.pylori* 23S rRNA, *E.coli* bases 2058 and 2059 are equivalent to *H.pylori* positions 2142 and 2143, respectively (see Fig. 1). Recently, Hunlten et al. described associated mutations at two additional positions (G2115 to A and G2141 to A) (120).

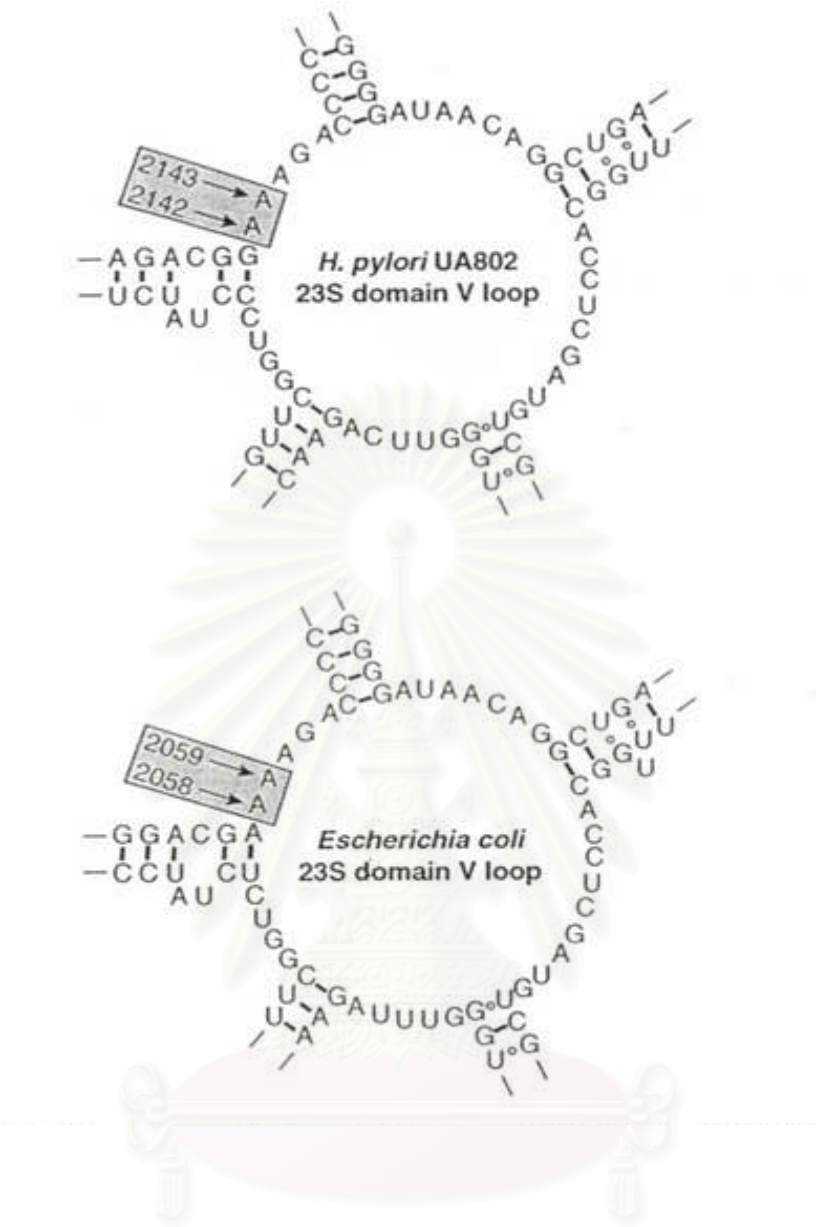


FIG. 1. secondary structure of the central part of domain V (peptidyltransferse loop) of the *H.pylori* 23S rRNA genes based on the model of Egebjerg et al. (121), with indication of the mutations that confer MLS resistance. The mutation sites are numbered according to the newly proposed numbering system (42), and the equivalent positions in *E.coli* are indicated in parentheses. The base substitutions made by site-directed mutagenesis in this work are indicated by arrows, and the associated MLS phenotypes are indicated.

In *E.coli*, as well as in some other bacteria, it is well known that the base equivalent to base A2058 in the 23S rRNA of *E.coli* is the target of ribosomal methyltransferase and the binding site for macrolide antibitotics (119, 122). Methylation or mutation at this position conferes complete cross-resistance to the macrolide, lincosamide, and type B streptogramin (MLS) antibiotics (MLS resistance), suggesting that these structurally distinct antibiotics have similar effects in inhibiting ribosomal function. Mutations within the vicinity, at position 2059 or 2057, have also been associated with resistance to the macrolide group of antibiotics (123, 124, 125). To date, the MLS resistance phenotypes associated with mutations in the peptidyltransferase region of the 23S rRNA have not yet been investigated in *H.pylori*.

6. Method for detection of clarithromycin resistance

6.1 Conventional method

6.1.1 Epsilometer test (E test)

This novel system for testing MICs represents a different approach from quantitative testing of antimicrobial activity and has some advantages over conventional methods in its simplicity, ease and robust nature. The commercial E test strips are 50-mm by 5-mm plastic carriers with an exponential antimicrobial gradient dried on one side and a graduated MIC scale on the other. To set up an E test, inoculate agar medium by flooding or with a swab in the same way that plates are inoculated for disc diffusion susceptibility tests. On incubation, elliptical zones of inhibition are produced and the MIC is read directly from the graduated E strip at the point of intersection of the zone of inhibition with the strip.

6.1.2 Agar dilution method

The agar dilution method for determining MICs has been accepted as the standard against which other methods are assessed. It has advantages over broth dilution methods in that contamination is more easily seen and reisolation of the required organism is usually not a problem.

6.2 molecular method

H.pylori is a relatively fastidious and slow growing microaerophilic microorganism and therefore standard culture based in vitro antibiotic susceptibility tests (disk diffusion, agar dilution, and Epsilometer-test methods), even in the hands of experts, are slow and can take at least 10-14 days from initial receipt of the gastric biopsy to reading and reporting the sensitivity results.

The introduction of molecular methods for *H.pylori* antibiotic resistance testing has been facilitated by the fact that the mode of action of clarithromycin is well understood and is due to various point mutations in the peptidyltransferase region of domain V of the 23S rRNA gene. *H.pylori* has two copies of that gene and the mechanism of resistance to clarithromycin appears to be decreased ribosome binding of the macrolide so that it fails to act by interrupting protein biosynthesis. In vitro determination of resistance to clarithromycin by conventional phenotypic tests generally gives reproducible results with a clear cut off point and so provides an accurate basis for validation of molecular assays. Isolates are considered to be resistant when the minimum inhibitory concentration (MIC) is >2 μg/ml and inter-laboratory reproducibility appears to be good even though the precise MIC value used may vary between laboratories.

Molecular assays for detecting clarithromycin resistance in *H.pylori* are all based on detection of mutations in the 23S rRNA genes. The basis assay, first described in 1996 (131), utilizes a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) approach in which the region of the gene containing the mutations is amplified and then digested with restriction endonucleases that cut specifically at the mutation sites. The sizes of the resultant fragments indicate the presence/absence of a particular mutation-for example, the presence of the A2142G mutant creates an *Mbo*II site giving two comigrating fragments of about 700 bp whereas the giving major fragments of about 700 and 300 bp. This basic form of assay has been used successfully to test isolates of *H.pylori* from patients in a number of different countries and the association between resistance phenotype and the presence of specific mutations has been validated by direct sequencing (41, 45, 120, 126).

Further interest in this molecular approach was stimulated by the finding from several groups of worker at that A2142G mutation was significantly more likely to be associated with higher levels of clarithromycin resistance (117, 127) which might be attributable to different steric effects of the mutations at the binding sites.

A significant development was the discovery that the PCR-RFLP assay could be successfully applied to evaluation of clarithromycin resistance without culture by direct analysis of DNA extracted from gastric juice (125) and from gastric biopsies (129, 130). Although such an approach is significantly faster with same day results, a feasible possibility, there is a risk that PCR efficiency may be affected by the presence of inhibitors from the clinical specimens and that specificity is reduced by the presence of high levels of non-*H.pylori* DNA, notably mammalian host DNA.

DEVELOPMENT OF IMPROVED ASSAYS FOR CLARITHROMYCIN RESISTANCE

The basic PCR-RFLP assay for detection of the two commonest A to G mutations has undergone various modifications to improve speed and specificity. For instance, a 3' mismatched PCR using an additional specific primer was applied to detect the rarer A2142C mutation (129). Alternative restriction enzymes have been used, notably *Bbs*I instead of *Mbo*II to improve detection of the A2142 nutation (129, 131). More important have been the developments of alternative formats to improve detection of specific mutations, such as the various hybridization assays using oligo probes for extracted DNA and in situ hybridization. Many of the assays are based on the principle of reverse hybridization with labeled probes for up to seven mutations and the wild type, immobilized either in microtitre wells (DEIA) or on nitrocellulose (LiPA). In these assays, PCR products were hybridized to the probes at highly stringent conditions and the resultant hybrids detected colorimetrically. In the DNA enzyme immunoassay (DEIA), the detection system was an enzyme linked immunoabsorbent assay with a labeled anti-double stranded DNA monoclonal antibody. Biotinylated probes for the wild type and three mutations (A2142C, A2142G, and A2143G) were designed to test for mutations in DNA extracted from cultures of *H.pylori* (142) and for use in a rapid (one day) laboratory assay that could be applied directly to gastric biopsies so avoiding the need for culture (132).

Table 5. Molecular methods for detection of point mutations associated with clarithromycin resistance in *Helicobacter pylori* (133).

PCR-restriction fragment length polymorphism (RFLP) (41)
PCR oligonucleotide ligation assay (OLA) (116)
DNA enzyme immunoassay (DEIA) (131)
PCR line probe assay (LiPA) (143)
PCR preferential homoduplex formation assay (134)
Real time PCR hybridization assay using the LightCycler
DNA sequencing technique

6.2.1 The polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a technique for the *in vitro* amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. DNA polymerase carries out the synthesis of a complementary strand of DNA in the 5′ to 3′ direction using a single-stranded template, but starting from a double-straned region. This is the primer extension reaction (figure 2) and is a basis for a variety of the labeling and sequencing techniques. The cycle, which only takes a few minutes, is repeated many times so that after many cycles there may be a million-fold replication of the target DNA (Figure 3) (135).

Figure 2.. Primer extension. DNA polymerase extends a primer by using a complementary strand as a template (135)

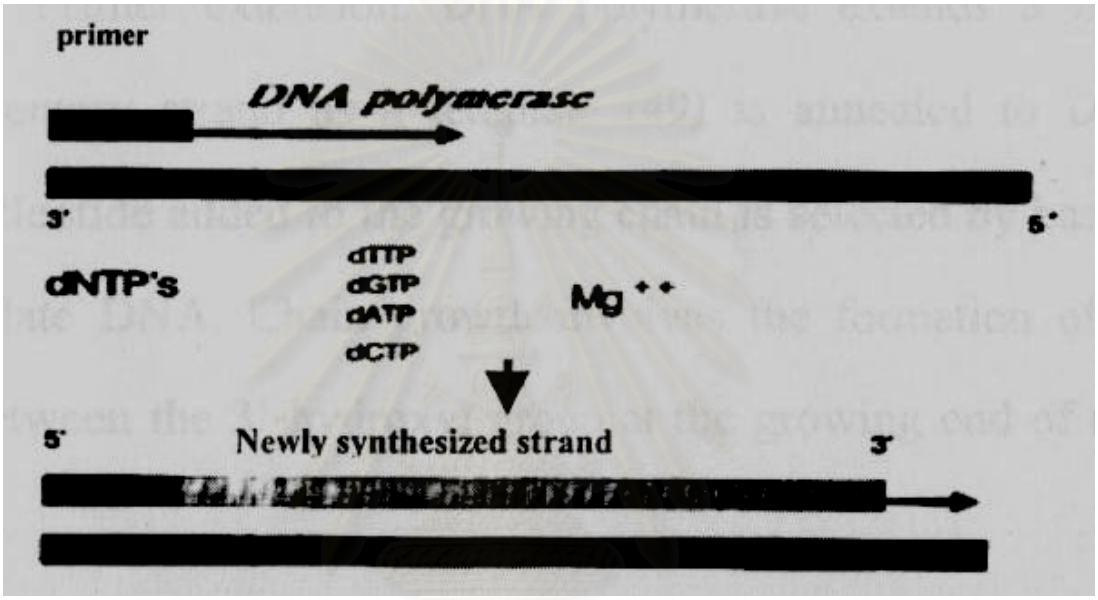
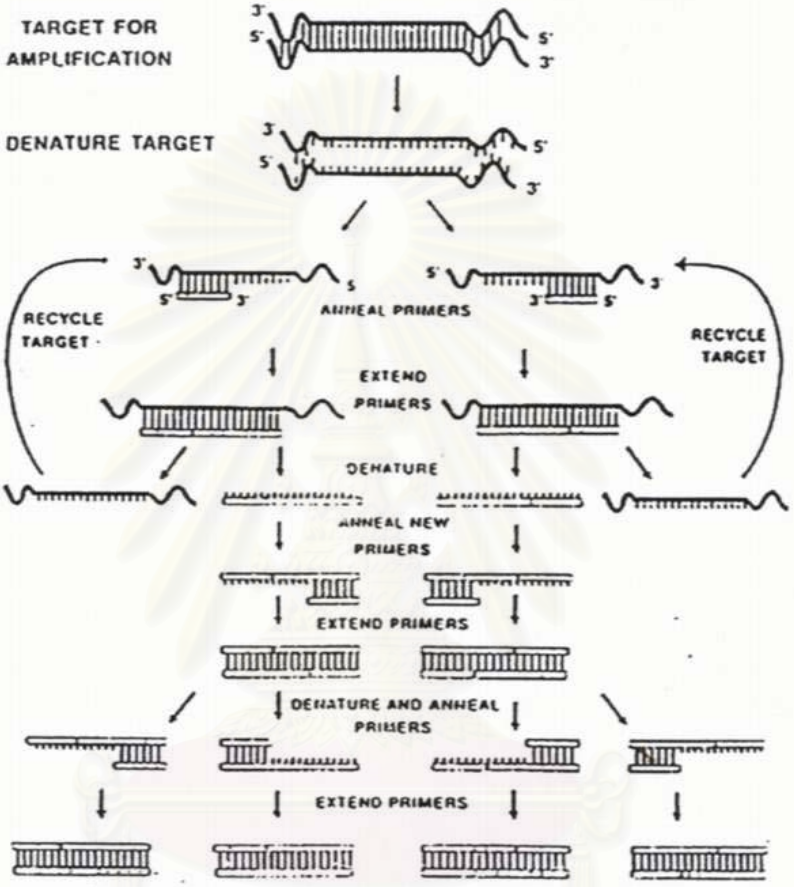


Figure 3. Schematic diagram of PCR (136)



6.2.2 DNA sequencing (Dideoxy sequencing) (137)

The dideoxy enzymatic method as originally developed by Sanger utilizes *E.coli* DNA polymerase I to synthesize a complementary copy of a single-stranded DNA template. After primer Figure 2 primer extension. DNA polymerase extends a primer by using a complementary strand as a template (136) is annealed to DNA template, the deoxynucleotide added to the growing chain is selected by base-pair matching to the template DNA. Chain growth involves the formation of a phosphodiester bridge between the $3'$-hydroxyl group at the growing end of the primer and the $5'$-phosphate group of the incoming deoxynucleotide. Overall chain growth is in the $5' \rightarrow 3'$ direction.

The Sanger sequencing method capitalizes on the ability of E. coli DNA polymerase I to use $2', 3'$-dideoxynucleotides as a substrates. When a dideoxynucleotide is incorporated at the $3'$ end of the growing primer chain, chain elongation is terminated selectively at G, A, T, or C because the primer chain now lacks a $3'$-hydroxyl group (figure 4) (137).

In an automate sequencer; thermal cycle sequencing is a method of dideoxy sequencing in which a small number of template DNA molecules are repetitively utilized and generate a sequencing ladder, A dideoxy sequencing reaction mixture (template, primer, dNTPs, ddNTPs, and a thermostable DNA polymerase) is subjected to repeated rounds of denaturation, annealing, and synthesis steps, similar to PCR using a commercially available thermal cycling machine (138). In practice, automate sequencing that uses fluorescent-based chemistry can provide accurate sequence data within 24-48 hours. (Figure 5).

Figure 4. Principle of DNA-sequencing method developed by Sanger. (137)

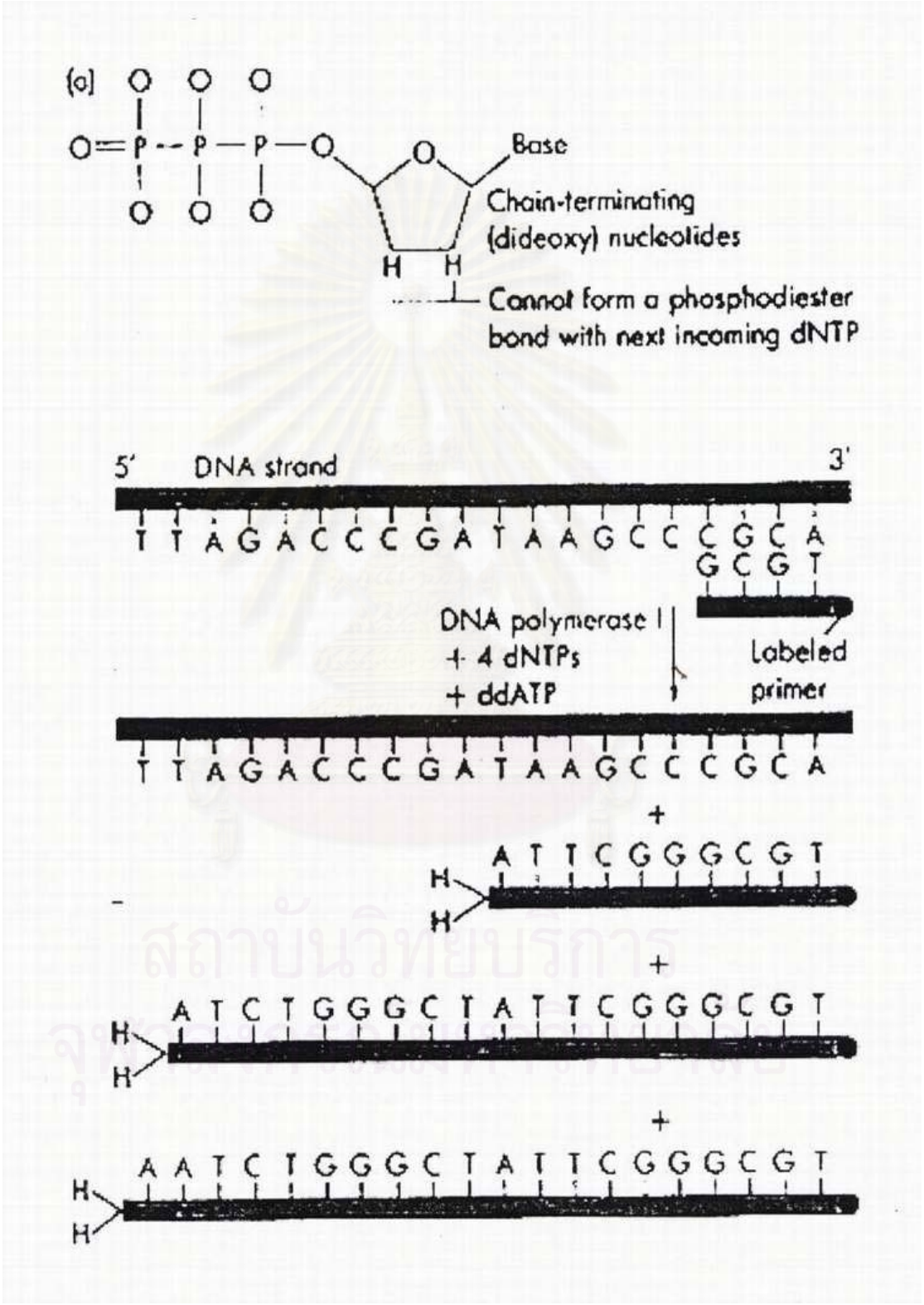
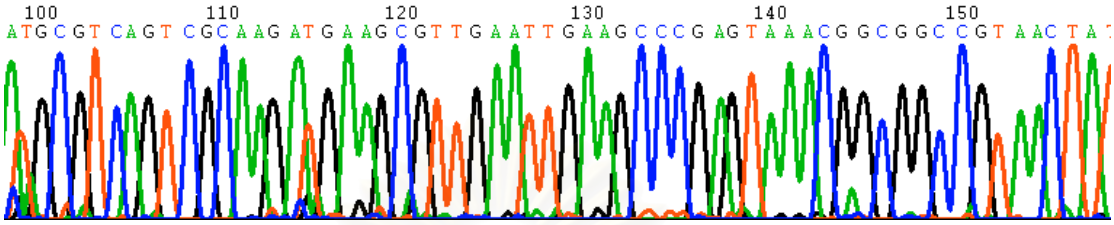


Figure 5. Chromatogram of sequencing by automate sequencer



6.2.3 Hybridization

Nucleic acid hybridization tests for the detection of specific DNA and RNA sequences are now extensively used in research and routine laboratories (139, 140, 141). Labeled nucleic acid probes are utilized in a variety of assay formats including dot blots, Southern blots (DNA target), Northern blot (RNA target), *in situ* hybridization, plaque hybridization and colony hybridization. An important aspect of nucleic hybridization assays is the choice of the substance used to label a nucleic acid probe and the label detection method. The first assays used a radioactive ^{32}phosphorus label. However, this label has the major disadvantage of a relatively short half-life (14.2 days). Many different substances have been tested as nonisotopic replacement for ^{32}phosphorus.

Nucleic acid labels

The majority of the substances used as labels for nucleic acid hybridization probes have been tested previously in immunoassay. Nonisotopic labels have been the focus of development because of the limitations of radioactive labels such as ^{32}phosphorus (142). These limitations are principally (1) a short-life that restricts the shelf life of labeled probes and hence hybridization assay kits, (2) possible health hazards during preparation and use of the labeled nucleic acid and (3) disposal of radioactive waste from the assay.

Enzymes, such as horseradish peroxidase and alkaline phosphatase, have become particularly popular in recent years as a range of sensitive detection methods has evolved. Alkaline phosphatase, for example, can be detected using chemiluminescent, bioluminescent and time-resolved fluorescent methods.

Horseradish peroxidase : the enzyme horseradish peroxidase (HRP) catalyzes the chemiluminescent oxidation of luminol and in the presence of small amounts of certain phenols (*para*-iodophenol), naphthols (1-bromo-2-naphthol) and amines (*para*-anisidine) (*enhancer*). The intensity of the light emission is increased by several orders of magnitude and background light emission from the luminol-peroxidase assay reagent is greatly reduced, which leads to a dramatic increase in the signal : background ratio.

Nucleic acid labeling procedures

Detection of probe : nucleic acid target hybrids can be accomplished by direct or indirect labeling methods. In the former case, a label is attached directly to the nucleic acid by a covalent bond or the label intercalates noncovalently between the double strand of the probe : nucleic acid target complex. The latter method, indirect labeling, employs a hapten (e.g., biotin) attached to the nucleic acid probe. The hapten is detected using a labeled specific binding protein (e.g., antibiotin, avidin or streptavidin) (Table 6.). A in one design, a biotin-labeled probe is hybridized to the target DNA, followed by reaction of the biotinylated probe with streptavidin. The remaining binding sites on tetravalent streptavidin are then reacted with a biotinylated poly (alkaline phosphatase) to obtain a cluster of alkaline phosphatase labels around the bound biotinylated probe (143).

Table 6. Indirect Labels for Nucleic Acid Hybridization Assays.

Hapten	Binding protein	Label
Biotin	Antibiotin	Gold colloid
	Avidin	Alkaline phosphatase
		Fluorescine
		Horseradish peroxidase
	Streptavidin	β-Galactosidase
Digoxigenin	Antidigoxigenin	Alkaline phosphatase
Ethidium	Antiethidium-DNA	β-Galactosidase
Glucosyl	Concanavilin A	Acid phosphatase
		Glucose oxidase
IgG	Antispecies IgG	Horseradish peroxidase
IgG, Fab fragment	Antispecies IgG	Horseradish peroxidase
Poly (dA)	Poly (dT)-DNA	Horseradish peroxidase
Poly (dT)	Poly (dA)-DNA	Horseradish peroxidase
Protein A	IgG	Horseradish peroxidase
Protein G	IgG	Horseradish peroxidase

Detection of Nonisotopic Labels

The principle types of detection methods for nonistopic labels are bioluminescence, chemiluminescence, colorimetry, electrochemiluminescence, fluorescence and time-resolved fluorescence.

1. Chemiluminescence and Bioluminescence

Chemiluminescence is the emission of light that occurs in certain chemical reactions because of decay of chemiexcited molecules to the electronic ground state. Bioluminescence is the chemiluminescence of nature, which involves luciferin substrates and luciferase enzymes, or photoproteins. Both methods are very sensitive, rapid and versatile.

2. Colorimetry

Colorimetry assays produce soluble colored products and are relatively insensitive compared to luminescent assays (chemiluminescence, fluorescence, etc.). Most attention has focused on reactions to produce insoluble colored products for locating hybrids on solid phases. These have the advantage of a simple visual read-out and a permanent record, especially for membrane-based assays.

3. Electrochemiluminescence

In this process, an electrochemical reaction produces excited-state species that decay to produce a ground state product and light. A disadvantage of this detection reaction is the need for specialized equipment that combines electrochemical-generation and light-detection capabilities.

4. Fluorescence and Time-Resolved Fluorescence

Fluorescence measurements are capable of detecting single molecules of fluorescein (112). In practice, however, fluorescence is plagued by background signal due to nonspecific fluorescence present in biological samples. Since the background fluoresce tened to be short-lived, it can be avoided by using a long-lived fluorophore that is

excited by a rapid pulse of excitation light. The fluorescence emission is then measured after the short-lived background has decayed, thus eliminating interference.

Several authors have described DNA-based diagnostic methods that offer a rapid alternative approach for macrolide susceptibility testing (41, 116, 127, 131, 132, 145). Recently, van Doorn et al. (145) evaluated a PCR-based reverse hybridization system (research prototype kit INNO-LiPA for *H.pylori* resistance) for the simultaneous detection of 23S rRNA point mutations. PCR products were analyzed by reverse hybridization in a reverse-hybridization LiPA. This assay is based on hybridization with a number of oligonucleotide probes that are immobilized as parallel lines on a nitrocellulose strip. *H.pylori* strains were tested by PCR-LiPA, DNA sequencing, RFLP and/or hybridization with oligonucleotide probes. The results were highly concordant, but PCR-LiPA appears to be more sensitive for the simultaneous detection of multiple mutants. This PCR method provides accurate information about different 23S rRNA mutants, even if they represent only a small portion of the bacterial population. Therefore, it could be particularly suitable for monitoring the development of resistance during antibiotic therapy.

Stone et al. (116) have developed an assay based on PCR followed by the oligonucleotide ligation assay (OLA) for the rapid detection of point mutations within the 23S rRNA gene of *H.pylori* strains. PCR-OLA discriminates sequence variations (transitions, transversions and deletions) by construcing probes to identify the variation of interest. This is different from using restriction enzymes to identify point mutations, because restriction enzymes are limited to sequence specificity. The authors have used PCR-OLA to determine the prevalence of 23S rRNA gene mutations. Susceptible *H.pylori* isolates were wild type at position 2143 and 2144, while 93% of the resistant isolates contained A-to-G mutations at either position and 7% of the isolates contained A-to-C mutations at position 2143. The MIC for 86% of the resistant isolates with an A2143 mutation was ≥ 64 μg of clarithromycin per ml and that for 89% of the resistant isolates with an A2144 mutation was ≤ 32 μg/ml. The detection of resistance marker, which could potentially be performed directly on biopsy material, could help to direct the treatment regimen for the patient, i.e., to use or not to use macrolides in therapy.

In the study by Occhialini et.al. (41), a PCR amplification with consecutive sequencing or RFLP was used. These authors used RFLP to detect the mutations in the 23S rRNA genes without sequencing by analyzing the occurrence of special restriction sites. These techniques have been used also by others. Maris et al. (132) and Pina et al. (131) have used a colorimetric hybridization in the liquid phase to detect the mutation at the molecular level after PCR amplification. All of the techniques applied for the detection of point mutations can be used with biopsy specimens or infected gastric juice.

CHAPTER IV

MATERIALS AND METHODS

1. Patients and clinical specimens

A total of 150 pateints (median age, 45 years; range 16 to 89 years) presenting upper gastrointestinal symptoms (peptic ulcer, gastritis and dyspepsia) were enrolled in the study, and all patients gave informed consent. All patients had not been submitted to eradicatation therapy in the previous 2 months.

All patients enrolled in the study underwent upper gastrointestinal endoscopy. Two biopsy specimens for each patient taken from the antrum and the gastric body using a disinfected endoscope, were placed in 0.1 ml of transport medium and sent to the Department of microbiology, Faculty of Medicine, Chulalongkorn University. The specimens were obtained during 19 months period between May 2001 to November 2002. The total number of specimens from patients having gastritis, dyspepsia and peptic ulcer was 58, 50 and 42 respectively.

A reference strain of *H.pylori* 3428 was kindly provided by Dr. Yoshio Yamaoka, Department of Medicine, Baylor College of Medicine, Houston, Texas, U.S.A. This reference strain was clarithromycin- resistant with MIC of 256 μg/ml.

2. Culture and identification method

Gastric biopsy specimens were inoculated on both selective medium brain heart infusion agar with antibiotics (vancomycin and amphotericin B) and 7% sheep blood and brain heart infusion agar without antibiotic and 7% sheep blood. The plates were incubated at 35°C to 37°C for 48-72 hours in an anaerobic jar (BBL Microbiology Systems, Cockeysville, Md.) under microaerobic atmosphere. The plates were visually inspected for small, translucent and glistening with a convex elevation and an entire edge

colonies. Suspected colonies were subjected to definitive identification by biochemical reaction tests as follows :

3.1 Urease test

A. Inoculum

Active growing colonies from solid media (fresh test specimen)

B. Reagent

Urea test broth and store at 4°C

C. Procedure

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

D. Results

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

3.2 Catalase test

A. Inoculum

Active growing colonies from solid media (fresh test specimen)

B. Reagent

- Hydrogen peroxide (3%) stored at room temperature
- sterile loop
- clean glass slide

C. Procedure

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

portion of the growth. The production of bubbles of gas was observed.

D. Result

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

3.3 Oxidase test

A. Inoculum

Active growing colonies from solid media (fresh test specimen).

B. Reagent

Oxidase reagent (tetramethyl-*p*-phenylenediamine dihydrochloride)

C. Procedure

A portion of the colony or subcultured growth to be tested was picked up and rubbed directly onto a portion of a reaction area of the dry slide oxidase. The reaction area was examined for appearance of a dark purple color within 20 sec.

D. Result

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

4. Clarithromycin susceptibility testing

All *H.pylori* isolates were tested for clarithromycin susceptibility by the epsilometer test (E-test) and agar dilution method.

4.1 E-test

A. Material required

- Mueller-Hinton Agar plates (150 mm) with 5% sheep blood media, sterile saline for inoculum preparation
- Sterile loops, swabs, test tubes, pipettes, forceps and scissors
- McFarland No.2 turbidity standard
- Incubator (37°C)

- *H.pylori* 3428 as quality control organism

B. Preparation of inoculum

An appropriate number of well isolated colonies were emulsified to ensure pure culture in saline or BHI broth to achieve a turbidity equivalent to a No.2 McFarland standard.

C. Procedure

E-test (AB Biodisk, Solna, Sweden) determinations of MICs were performed on Mueller-Hinton agar plates (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 5% sheep blood. A sterile swab was dipped into a bacterial suspension equivalent to a No. 2 McFarland standard. After swabbing the entire plate surface with inoculum, sterile E-test strips, impregnated with clarithromycin ranging in concentration from 0.016 to 256 μg/ml, were placed on the agar surface. Plates were incubated at 37°C under microaerobic conditions for 3 to 4 days. After the required period of incubation and when bacterial growth became distinctly visible, the MIC value was read at the point of intersection between the zone edge and the E-test strip. For *H.pylori*, two zones of inhibition are commonly seen, the second or higher zone is read as the MIC. Isolates were classified as clarithromycin resistant if the MIC exceeded >1 μg/ml (108, 109).

4.2 Agar dilution method

A. Material required

- Mueller-Hinton agar plate (150 mm) with 5% sheep blood media, sterile saline for inoculum preparation
- Sterile loops, swabs, test tubes, and pipettes
- McFarland No.2 turbidity standard
- Incubator (37°C)
- *H.pylori* 3428 as quality control organism

B. Preparation of inoculum

An appropriate number of well isolated colonies were emulsified to ensure pure culture in saline or BHI broth to achieve a turbidity equivalent to a No.2 McFarland standard.

C. Procedure

In this study, strains determined to be clarithromycin resistant were confirmed by the agar dilution procedure approved methodology recommended by the NCCLS (96, 97). Isolates were classified as clarithromycin resistant if the MIC exceeded 1 μg/ml.

The compounds were dissolved and diluted according to the recommendations of the manufactures and solutions were used on the day of preparation. The agar plates used for the agar dilution study contained twofold dilutions of antibiotic ranging in concentration from 0.008 to 256 μg/ml.

5. DNA preparation (Extraction of DNA)

The DNA was purified according to the manufacturer's directions by using the QIAamp®DNA Mini kit (Qiagen Corporation, Germany). Briefly, 1 ml of stock culture in BHI-glycerol broth was pelletted by centrifugation for 5 minute at 5,000 x g. Bacterial cell lysis was performed by adding 180 μl ATL buffer and 20 μl Proteinase K to the cell pellet. After well mixing the suspension was incubated at 56°C until the pellet was completely lysed, 200 μl of the buffer AL were added. After well mixing the suspension was heated at 70°C for 10 min, the mixure was vortexed for at least 30 sec. The supernatant was then transferred into QIAamp spin column in a clean 2 ml collection tube, centrifuged at 6,000 x g for 1 min and the tube containing the filtrate was discarded. QIAamp spin column was carefully opened and 500 μl buffer AW1 were added. It was then centrifuge at 6,000 x g for 1 min and then was discarded. QIAamp spin column was then washed with buffer AW2 500 μl and centrifuged at 20,000 x g for 3 min. After centrifugation the QIAamp spin column was placed in a clean 1.5 ml microcentrifuge tube and 200 μl buffer AE were added and then incubated at room temperature for 1 min. After incubation, the suspension was centrifuged at 6,000 x g for 1 min and stored at -20°C, ready for the PCR analysis.

6. DNA amplification by polymerase chain reaction (PCR)

Polymerase chain reaction was performed by the method of Pina, et al. (131) with primers HP 23S-1 (5' CCA CAG CGA TGT GGT CTC AG 3', position 1820 to 1839, Gene Bank accession number U27270) and HP 23S-2 (5' CTC CAT AAG AGC CAA AGC CC

3', position 2244 to 2225, Gene Bank accession number U27270). The amplified product of the 23S rRNA gene was 425 bp. Amplification was performed in 50 μl mixture containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 1 U of *Taq* polymerase, 200 μM deoxynucleoside triphosphates (dNTPs, dATP, dCTP, dGTP, and dTTP) and 0.2 μM of each primer, and 50 μl of mineral oil (Sigma, St. Louis, Mo.) to prevent evaporation. The reaction was performed in 0.5 ml eppendorf tube with target DNA on a Hybaid OmniGene Thermal cycles. The PCR cycling parameters were 94°C for 10 min, followed by 40 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, extension at 72°C for 1 min, and one cycle of 72°C for 10 min.

7. Detection of amplification product

Ten microliters of PCR product were mixed with 3 μl of gel loading buffer (20% ficoll, 0.05% bromophenol blue), analyzed by electrophoresis on 1.5% agarose gel, consisted of 50 μg/ml ethidium bromide, in 1XTris-acetate-EDTA (1XTAE) buffer (pH 8.0). The electrophoresis was carried out at 80 volts for 30 min. Gel was visualized with UV transillumination, and the positive result of PCR showed a single band of 425 bp fragment for 23S rRNA gene compared with the 100 bp molecular size marker.

8. Sequencing of the 23S rRNA gene

An ABI PRISM Big Dye™ Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, Ca.) was used for the sequencing of the PCR product. Sequencing was performed on 30-90 ng of purified PCR product using QIAquick PCR Purification Kit; Qiagen Corporation, Germany. The sequencing reaction required 4 ml of Premix, 3.2 pmol of primer, and 150 ng of PCR product template in a total volume of 10 μl. The sequencing reaction was performed using Perkin Elmer GeneAmp PCR system 9600 with cycling parameters of 25 cycles of 96°C for 30 sec, 55°C for 10 sec, 60°C for 4 min and hold at 4°C until ready to purify. The product was purified with ethanol/sodium acetate precipitation before capillary electrophoresis was run for sequencing analysis.

The DNA sequence was compared to published sequences of 425 bp region of 23S rRNA gene in *H.pylori* in the GenBank accession number U27270.

9. Reverse dot blot hybridization

9.1 Preparation of the reverse dot blot

The Biodyne C membranes were prehybridized in 10 ml freshly prepare 16% (w/v) 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) in demineralized water at room temperature for 15 min, and the membranes were placed in plastic container and shaked with demineralised water for 2 min. After the membranes were activated, they were placed on support cuchsion in a clean dotblotter system. Dilutes of the 6 oligonucleotides solution in 100 µl 0.5 M $NaHCO_3$, pH 8.4 (Table 7) were added to the dot of dotblotter and incubated for 1 min at room temperature. After incubation, the oligonucleotide solutions were removed by suction and the membranes were removed from the dotblotter and incubated in 200 ml freshly prepared 0.1 M NaOH for 10 min at room temperature in a plastic container and rinsed with demineralised water. The membranes were washed in 50 ml 2XSSPE/0.1% SDS for 10 min at 50°C and finally washed in 50 ml 20 mM EDTA pH 8.0 for 15 min at room temperature.

Table 7. Sequences and concentration of the *H.pylori* amino-link oligonucleotides

Oligo no.	Type	Oligo name	Sequence (5' → 3')	Tm (°C)	concentration (pmole/100µl)
1.	wild type	clari 2143W	AGACGGAAAGACCCC	61.1	10
2.	wild type	clari 2182W	GCACTGCTAATGGGA	58.4	7.5
3.	wild type	clari 2223W	AGTAAGGGCTTTGGC	58.4	0.1
4.	mutant	clari 2143G	AGACGGAGAGACCCC	63.8	10
5.	mutant	clari 2182C	GCACTGCTAACGGGA	61.1	7.5
6.	mutant	clari 2223G	AGTAGGGGCTTTGGC	61.1	0.075

9.2 Reverse dot blot hybridization

All buffer should be prewarmed before use. Prepare the following buffers from concentrated stocks, using demineralized water for dilution (50 ml 2 X SSPE/0.1% SDS, 50°C, 100 ml 2 X SSPE/ 0.5% SDS, 55°C, 100 ml 2 X SSPE/0.5% SDS, 42°C, and 100 ml 2 X SSPE, room temperature). Forty microliters of the PCR products were added to 1500 µl 2XSSPE/0.1%SDS, and then heat-denatured for 10 min at 100°C and cool on ice immediately. The membrane was washed for 5 min at 50°C in 50 ml of 2 X SSPE/0.1% SDS. The membrane was then placed into hybridization bag and the bag was filled with 1500 µl of diluted PCR product (avoid air bubbles). Hybridization was performed with shaking for 45 min at 50°C. The membrane was then from hybidization bag using forcep and washed twice in 50 ml 2 X SSPE/0.5% SDS for 10 min at 57°C.

9.3 Detection of hybridization signals

The membrane with the hybridized PCR products was placed in a plastic container and allowed to cool down to prevent inactivation of the peroxidase in the next step. Solution of 2.5 µl streptavidin-peroxidase conjugate (500 U/ml) was added to 10 ml

of 2 X SSPE/0.5% SDS, and the membrane was incubated in this solution for 30 min at 42°C in the plastic container. The membrane was washed twice in 250 ml of 2 X SSPE/0.5% SDS for 10 min at 42°C to remove excess streptavidin-peroxidase, and rinsed twice in 250 ml of 2 X SSPE for 5 min at room temperature to remove SDS. For chemiluminescent detection of hybridized DNA, the membrane was incubated for 1 min in 20 ml ECL detection liquid (10 ml detection reagent 1 and 10 ml detection reagent 2). The membrane was placed on a carrier by using forceps and covered with a transparent plastic sheet and a light sensitive film was exposed to the membrane for 15 min. If the signal is too weak or too strong, the membrane can be used again to expose another film for a shorter or longer period.

CHAPTER V

RESULTS

1. Culture and identification

One hundred and fifty gastric biopsy specimens were obtained during the study period of 19 months. Colonies grown on brain heart infusion agar were examined for small, translucent and grey to yellow appearance. Suspicious colonies were gram-stained to look for curved or S-shaped Gram-negative rods. The presumptive phenotypic characteristics (urease test, oxidase test and catalase test) were tested with suspected bacterial isolates. A total of 71 (47.33%) out of 150 isolates were identified as the *Helicobacter pylori* based on the presumptive phenotypic characteristics. They were all positive for catalase test, oxidase test and rapid urease reaction.

2. Determination of the clarithromycin susceptibility of *H. pylori*

Of the 71 *H. pylori* isolates, 40 isolates (56.33%) were susceptible to clarithromycin (MIC, 0.016 to 0.5 μg/ml), and the remaining 31 isolates (43.66%) were resistant to clarithromycin (Table 8). Among these resistant strains, eleven isolates presented a high level of resistance (MIC, 128 to 256 μg/ml), while nineteen isolates showed a low level of clarithromycin resistance, being inhibited by clarithromycin at 1 μg/ml. No differences in MICs were found for all the resistant strains when these were tested by E-test as well as the agar dilution method (Table 8). *H.pylori* 3428 used for quality control the E test and agar dilution method gave MIC of 128 to 256 μg/ml.

Table 8. Number of clarithromycin- resistance isolates

No. of *H.pylori* isolated	No. of *H.pylori* resistant to clarithromycin tested by:		No. of *H.pylori* susceptible to clarithromycin tested by:	
	E test	Agar dilution	E test	Agar dilution
71	31	31	40	40

3. PCR amplification of 23S rRNA gene

Primers HP 23S-1 and HP 23S-2, designed to amplify 23S rRNA fragment from *H. pylori*, yielded a 425-bp PCR product. Amplicons were obtained for all the 71 *H. pylori* isolates examined (figure 6).

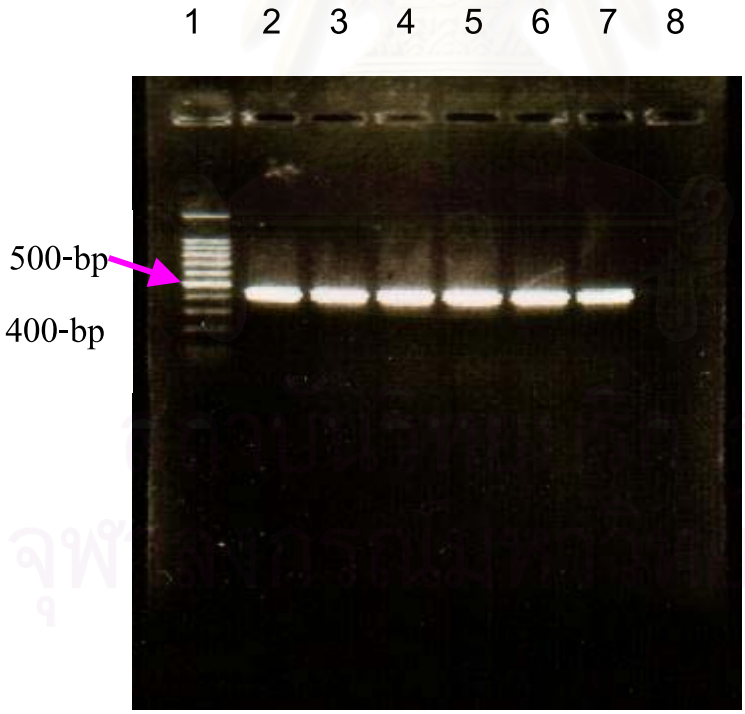


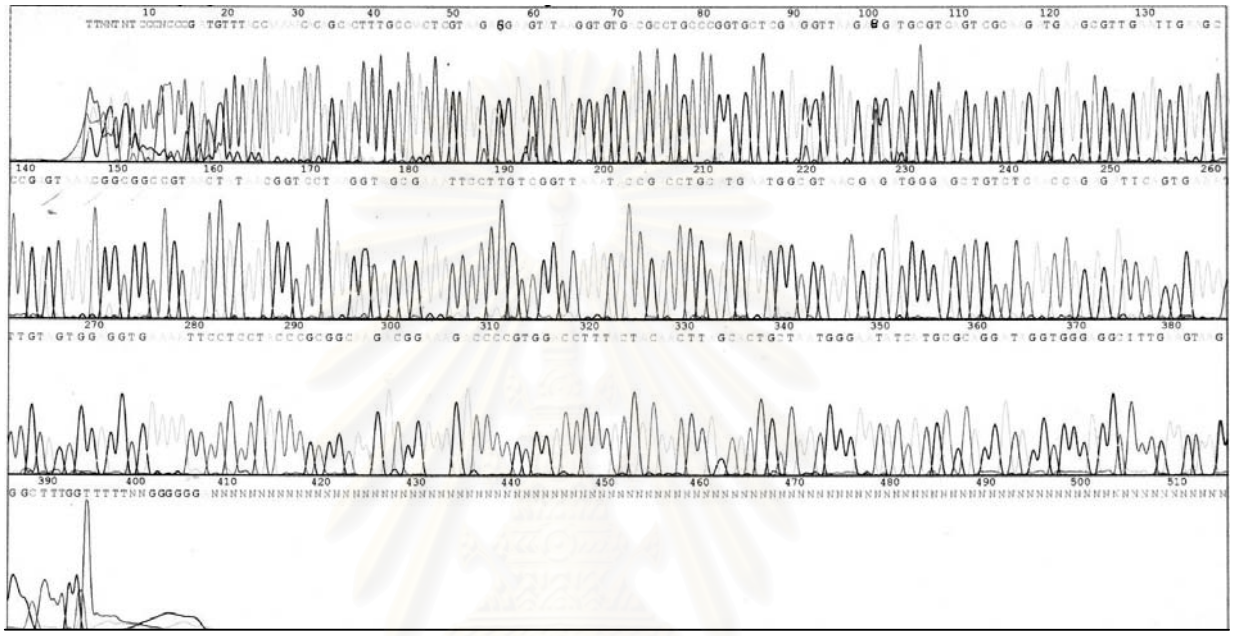
Figure 6. Amplification of *H. pylori* DNA. Lanes : 1, 100-bp DNA ladder; 2-6, *H.pylori* DNA from clinical isolates; 7, *H.pylori* DNA-positive control; 8, negative control

4. 23S rRNA sequencing results

To examine the genetic basis of these resistant phenotypes, all isolates were analyzed for the nucleotide sequence of the 425-bp PCR product of the 23S rRNA gene.

Our isolates showed the mutation described at positions 2143, 2182, and 2223. The sequence analysis of 30 resistant isolates, presented mutation point as follow : the transition of A to G at position 2143 (2 isolates), the transition of T to C at position 2182 (31 isolates) and the transition of A to G at position 2223 (3 isolates). In figure 7 the wild type strain, figure 8 was indicated by an arrow the position of A2143G mutation, figure 9 was indicated by an arrow the position of T2182C mutation, and figure 10 was indicated by an arrow the position of A2223G mutation. Sequencing results of all isolates were shown in Table 9.

Figure 7. Nucleotide sequence of the 425-bp amplicon from the 23S rRNA gene from *H. pylori*-susceptible isolate.



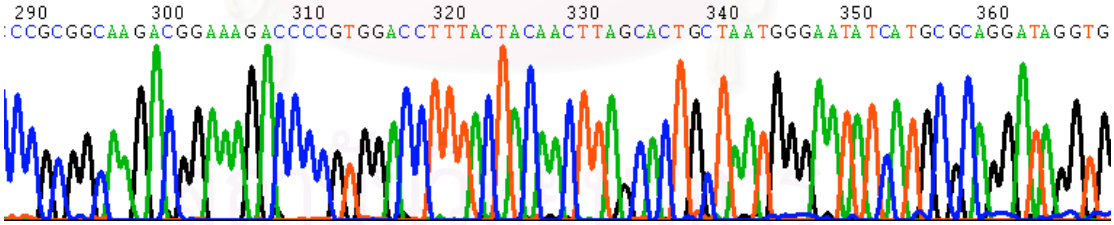
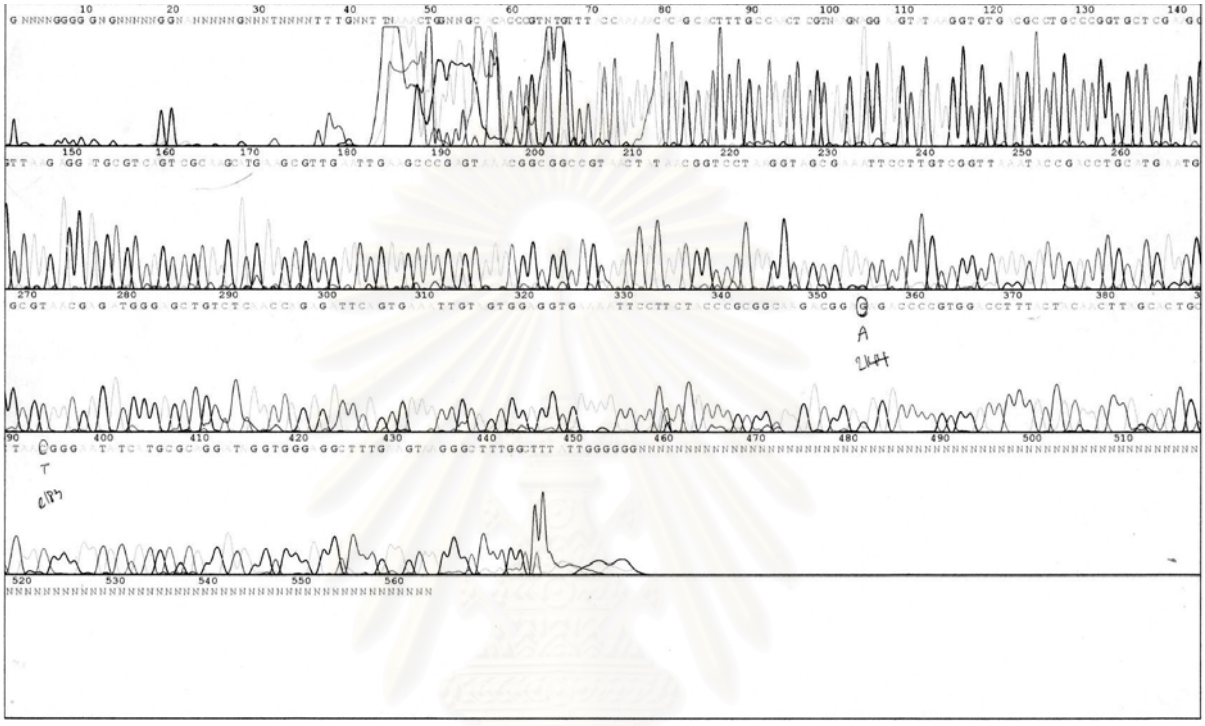
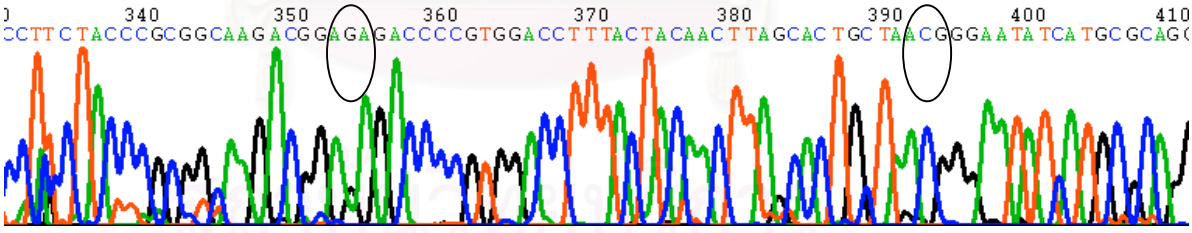
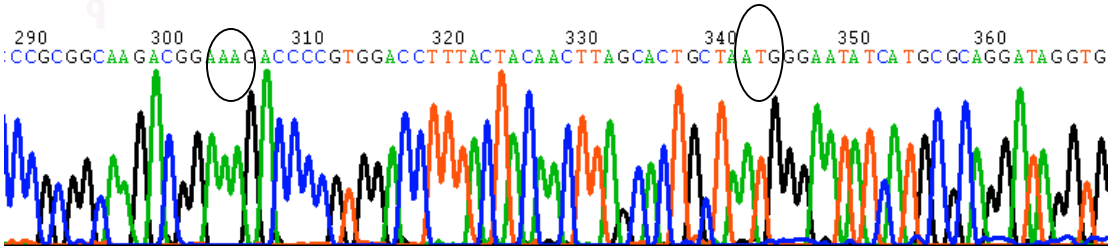


Figure 8. Nucleotide sequence of the 425-bp amplicon from the 23S rRNA gene from an *H. pylori*-resistant isolate. The circle indicate point mutations of A2143G transition and T2182C transition.



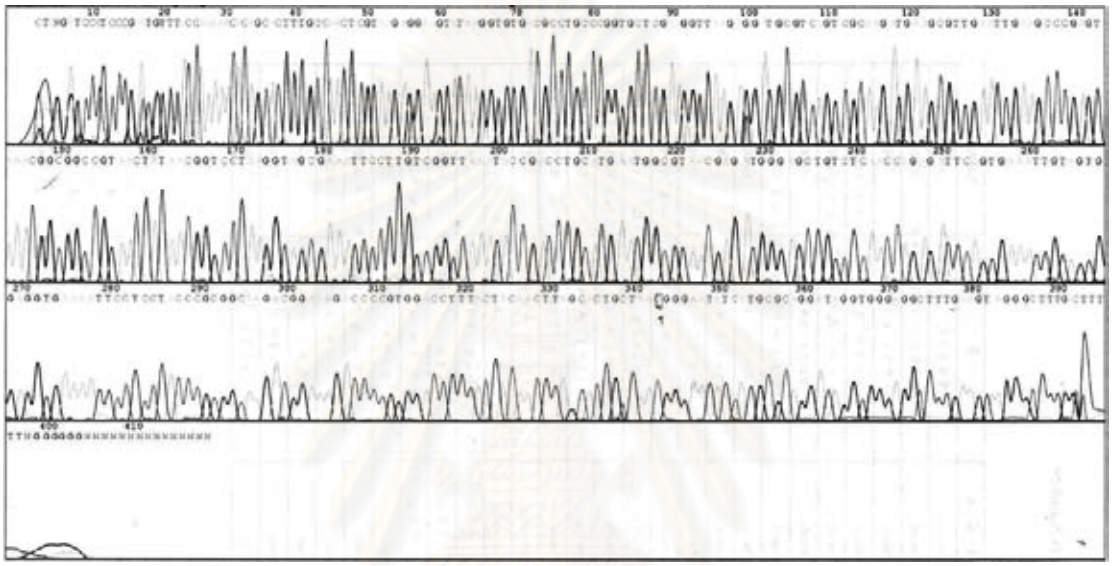


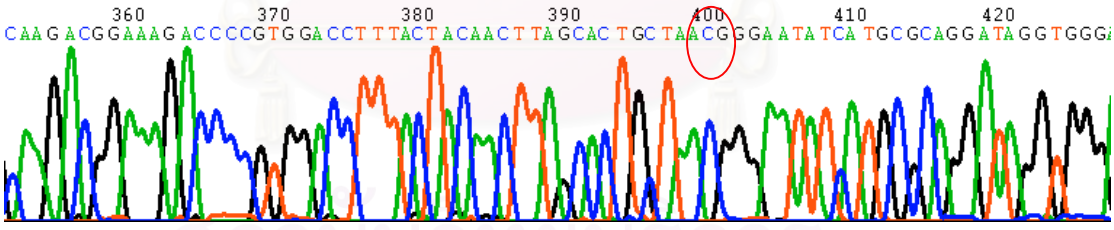
mutant



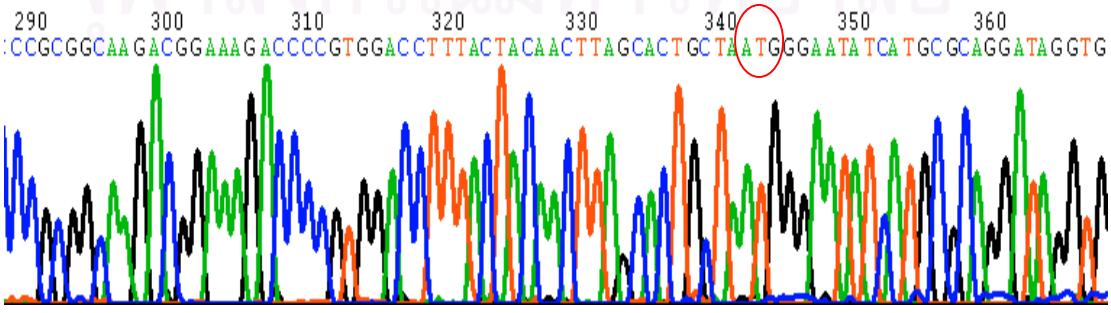
wild type

Figure 9. Nucleotide sequence of the 425-bp amplicon from the 23S rRNA gene from an *H. pylori*-resistant isolate. The circle indicate a single point mutation, that is, a T2182C transition.



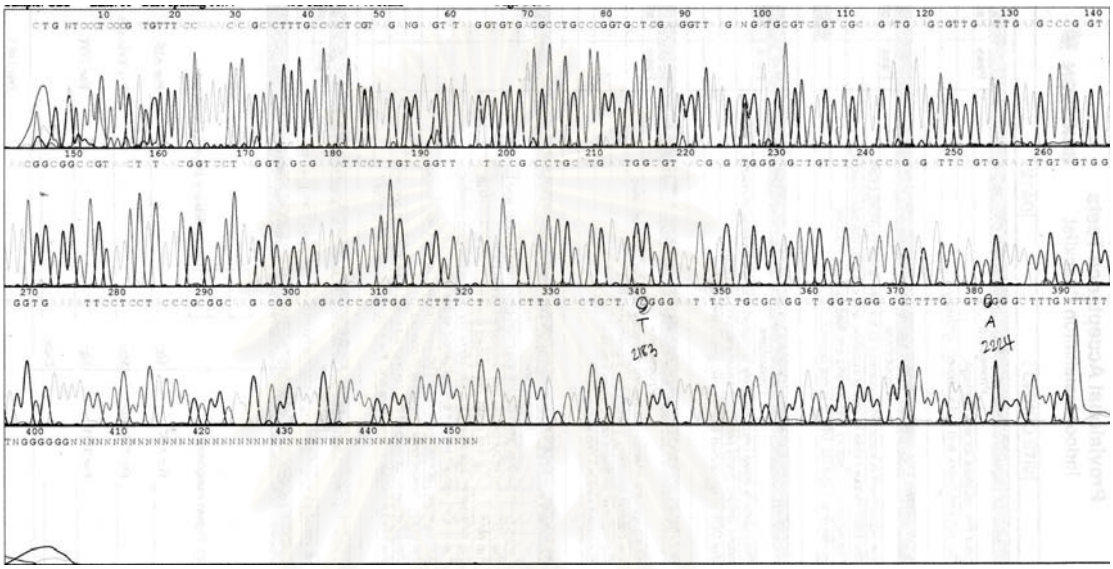


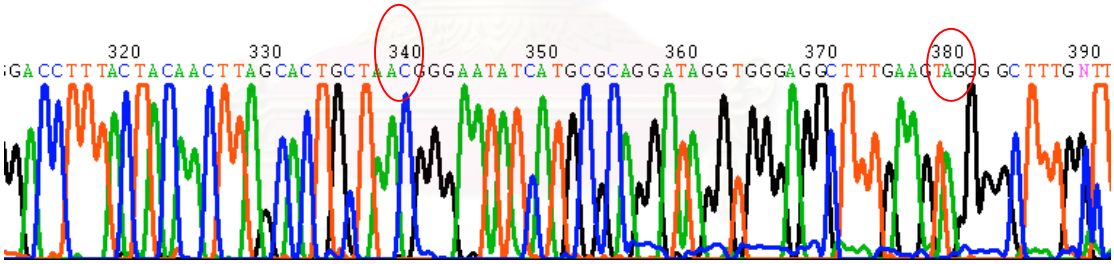
mutant



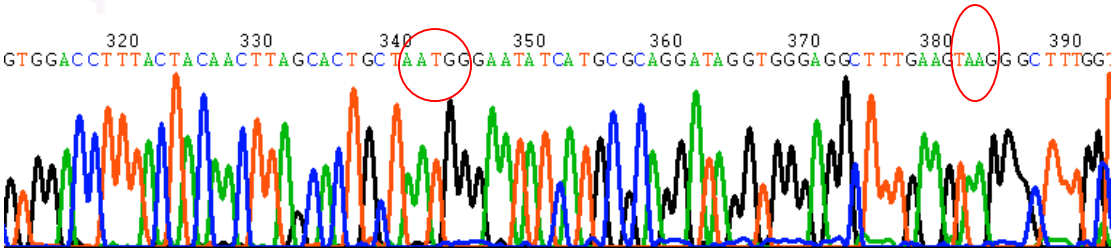
wild type

Figure 10. Nucleotide sequence of the 425-bp amplicon from the 23S rRNA gene from an *H. pylori*-resistant isolate. The circle indicate point mutations of A2223G and T2182C transition.





mutant



wild type

Table 9. Comparison of three methods for determination of the clarithromycin susceptibility of *H. pylori*

Number	Code	MIC by E-test	MIC by agar dilution	Mutation
1.	550G	0125 (S)	0.125 (S)	-
2.	94G	8 (R)	8 (R)	T2182C
3.	12A	2 (R)	2 (R)	T2182C
4.	102G	0.016 (S)	0.125 (S)	-
5.	13R	0.032 (S)	0.125 (S)	-
6.	114G	128 (R)	256 (R)	T2182C
7.	IL-51	0.016 (S)	0.25 (S)	-
8.	IL-31	256 (R)	256 (R)	T2182C
9.	IL-2	0.016 (S)	0.25 (S)	-
10.	17T	4 (R)	2 (R)	T2182C
11.	121G	0.019 (S)	0.125 (S)	-
12.	109G	0.47 (S)	0.25 (S)	-
13.	111G	0.016 (S)	0.25 (S)	-
14.	IL-3	0.016 (S)	0.125 (S)	-
15.	BRUSH 30	2 (R)	2 (R)	T2182C
16.	136G	256 (R)	128 (R)	T2182C

Table 9. (cont.) Comparison of three methods for determination of the clarithromycin susceptibility of *H. pylori*

Number	Code	MIC by E-test	MIC by agar dilution	Mutation
17.	160G	0.5 (S)	0.5 (S)	-
18.	155G	0.047 (S)	0.125 (S)	-
19.	167G	0.016 (S)	0.125 (S)	-
20.	178G	0.047 (S)	0.125 (S)	-
21.	BRUSH39	0.016 (S)	0.125 (S)	-
22.	IL-72	256 (R)	64 (R)	T2182C
23.	175G	4 (R)	2 (R)	T2182C
24.	1G	256 (R)	128 (R)	T2182C
25.	188G	256 (R)	128 (R)	T2182C
26.	IL-65	0.016 (S)	0.125 (S)	-
27.	190G	256 (R)	128 (R)	T2182C
28.	200G	2 (R)	2 (R)	T2182C
29.	208G	0.19 (S)	0.25 (S)	-
30.	204G	8 (R)	8 (R)	T2182C
31.	217G	0.047 (S)	0.25 (S)	-
32.	212G	0.016 (S)	0.125 (S)	-

Table 9. (cont.) Comparison of three methods for determination of the clarithromycin susceptibility of *H. pylori*

Number	Code	MIC by E-test	MIC by agar dilution	Mutation
33.	205G	0.125 (S)	0.125 (S)	-
34.	213G	256 (R)	256 (R)	T2182C, A2223G
35.	218G	3 (R)	4 (R)	T2182C
36.	IL-81	0.032 (S)	0.125 (S)	-
37.	GB2	1 (R)	2 (R)	T2182C, A2223G
38.	247G	3 (R)	2 (R)	T2182C
39.	248G	0.064 (S)	0.125 (S)	-
40.	250G	1 (R)	2 (R)	T2182C
41.	251G	0.032 (S)	0.25 (S)	-
42.	266G	0.023 (S)	0.25 (S)	T2182C
43.	270G	0.016 (S)	0.125 (S)	T2182C
44.	273G	24 (R)	32 (R)	T2182C, A2223G
45.	275G	2 (R)	2 (R)	T2182C, A2143G
46.	269G	2 (R)	2 (R)	T2182C, A2143G
47.	IL-100	0.38 (S)	0.5 (S)	-
48.	325G	1 (R)	2 (R)	T2182C

Table 9. (cont.) Comparison of three methods for determination of the clarithromycin susceptibility of *H. pylori*

Number	Code	MIC by E-test	MIC by agar dilution	Mutation
49.	360G	0.50 (S)	0.50 (S)	-
50.	N-21	0.023 (S)	0.0125 (S)	-
51.	IL-120	0.032 (S)	0.125 (S)	-
52.	IL-129	256 (R)	128 (R)	T2182C
53.	397G	0.016 (S)	0.25 (S)	-
54.	407G	0.016 (S)	0.25 (S)	-
55.	409G	0.016 (S)	0.125 (S)	-
56.	418G	0.50 (S)	0.5 (S)	-
57.	419G	0.016 (S)	0.125 (S)	-
58.	IL-87	0.016 (S)	0.125 (S)	-
59.	436G	0.023 (S)	0.25 (S)	-
60.	438G	0.125 (S)	0.125 (S)	-
61.	441G	0.032 (S)	0.125 (S)	-
62.	IL-133	0.047 (S)	0.125 (S)	-
63.	468G	256 (R)	128 (R)	T2182C
64.	470G	4 (R)	8 (R)	T2182C

Table 9. (cont.) Comparison of three methods for determination of the clarithromycin susceptibility of *H. pylori*

Number	Code	MIC by E-test	MIC by agar dilution	Mutation
65.	475G	0.016 (S)	0.25 (S)	-
66.	437G	0.025 (S)	0.125 (S)	-
67.	497G	256 (R)	256 (R)	T2182C
68.	522G	8 (R)	8 (R)	T2182C
69.	531G	0.025 (S)	0.125 (S)	-
70.	532G	0.016 (S)	0.25 (S)	-
71.	506G	4 (R)	2 (R)	T2182C

5. Reverse dot blot hybridization

All isolates (71 isolates ; 100%) tested by reverse dot blot hybridization yielded exactly the same result as sequencing method. (figures 11-14). All results for the summarized in table 10.

Figure 11. Representative example of reverse dot blot hybridization for detection of wild type. The positions of probes on the strip are shown. Isolate no. IL-65 is wild type.

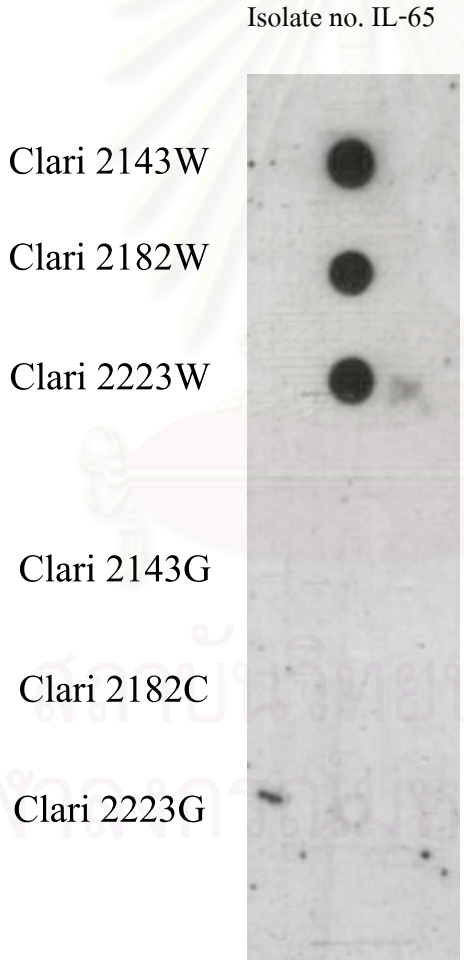


Figure 13. Representative example of reverse dot blot hybridization for detection of T2182C mutation. The positions of probes on the strip are shown. Isolat no.G1 contained T2182C mutation.

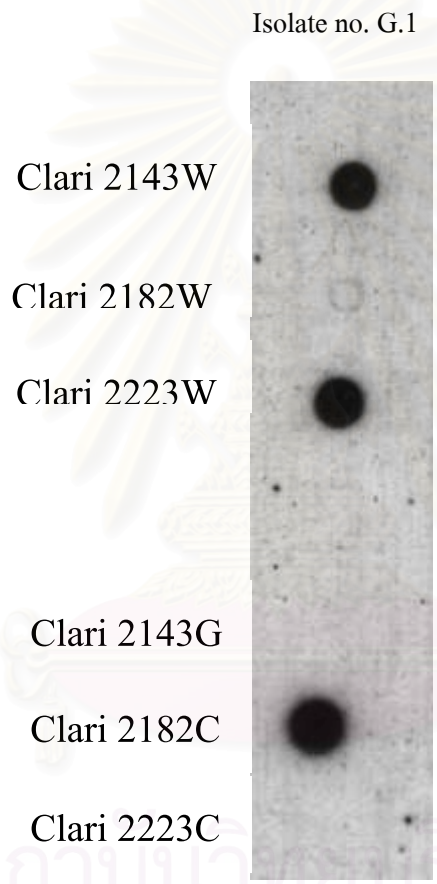


Figure 14. Representative example of reverse dot blot hybridization for detection of mutations at T2182C and A2223G. The positions of probes on the strip are shown. Isolated no.GB2 contained mutations at T2182C and A2223G mutations.

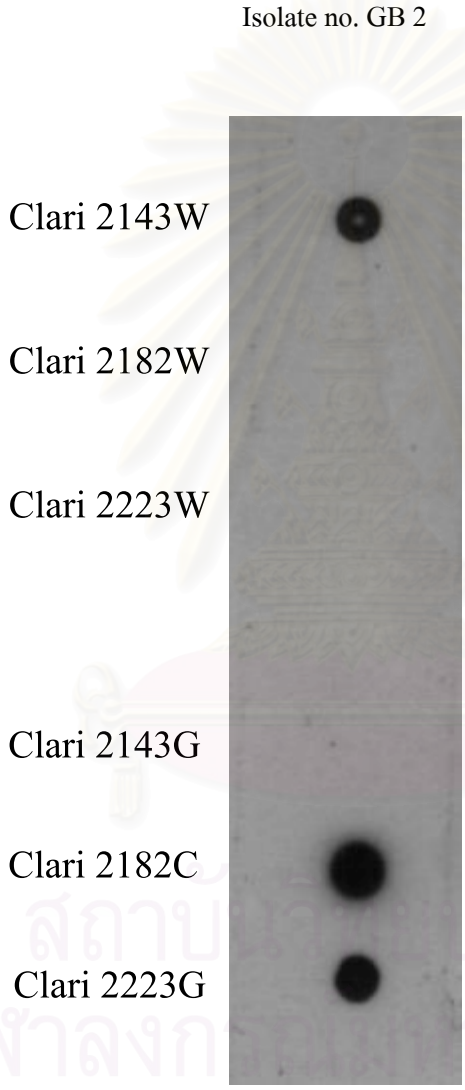


Figure 12. Representative example of reverse dot blot hybridization for detection of mutations at T2182C and A2143G. The positions of probes on the strip are shown. Isolate no. 269 contained mutations at T2182C and A2143G mutations.

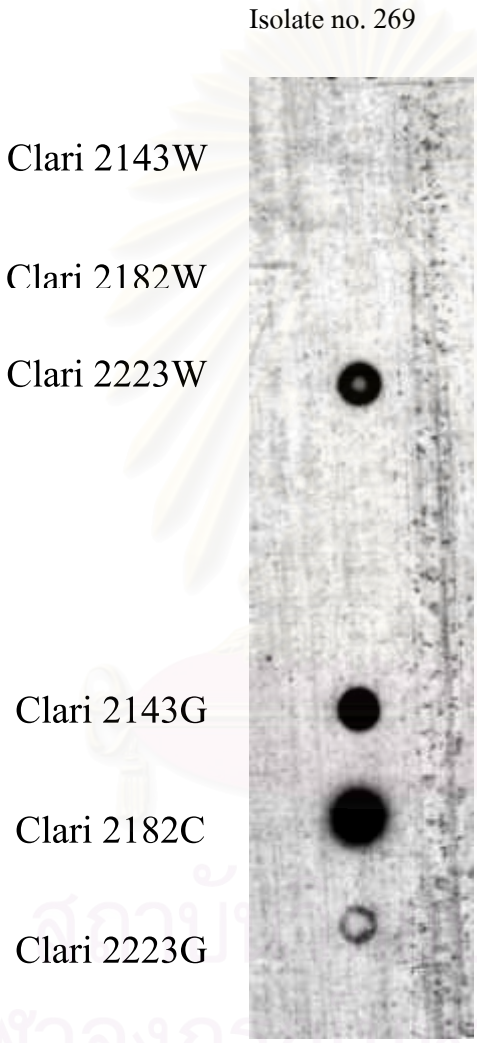


Table 10. Comparative results of sequencing and reverse dot blot hybridization methods for the detection of 23S rRNA mutations.

Mutation detection	Total no. of strains with mutation detected by hybridization					
by sequencing	wild type	A2143G	T2182C	A2223G	double mutations	total
Wild type	40					40
A2143G		2				2
T2182C			31			31
A2223G				3		3
double mutations					5	5

CHAPTER VI

DISCUSSION

Rapid and accurate detection of antibiotic resistance plays an increasing important role in the management of *H.pylori*-infected patients. Detection of resistance in *H.pylori* is generally performed by MIC determination methods such as agar dilution and E-test. This method requires growth of bacteria and takes at least 10 days; thus, the applicability of the data in the clinical setting is sometimes controversial. Due to the time-consuming of conventional susceptibility testing methods, molecular tools were applied to alleviate this problem. Molecular methods can be applied to *H.pylori* culture obtained from gastric biopsy specimens to detect mutation (s) in the gene associated with drug resistance.

In the present study, the results of conventional clarithromycin susceptibility testing were compared with those of molecular analysis by DNA sequencing and PCR-reverse hybridization. *H.pylori* isolates were tested by agar dilution and E-test. Since it is recommended to use both of these methods to identify clarithromycin resistance (144). DNA sequencing assay was employ to detect point mutation (s) associated with resistance on the amplified sequence of the 23S rRNA gene of *H.pylori*. DNA sequencing provides the gold standard reference method for mutation detection although it is not technically feasible or cost effective for rountine laboratory determination of *H.pylori* resistance markers. Sequencing is obviously the best approach to detect mutations but it is rather fastidious and time consuming even when automated sequencing is used.

The PCR- reverse hybridization method permits the same result as sequencing for detection 23S rRNA gene mutations. Of these, the T2182C mutations were the most prevalent and accounted for 100% of the clarithromycin-resistant strains containing single 23S rRNA genotype, the A2143G mutation was found in only two strains, and the A2223G mutation was found in only three strains.

Early studies of the genetic test of various *H.pylori* strains have demonstrated that *H.pylori* clarithromycin resistance is associated to a common single mutation point (119, 122). This mutation most frequently affects positions A2142G or A2143G in *H.pylori* (119, 122, 127) and less frequently positions A2142C, G2115A, G2141A, T2717C and T2182C (41, 120, 145, 146). The mutation (s) found in all resistant isolates at position 2182 in this study presents a mutation site that has been described by Kim et al. in 4 of 12 resistant strains. In addition, 3 isolates had mutation at position 2223 which has not been described before. Mutations in these positions are associated with *H.pylori* phenotypes expressing different levels of resistance, MIC ranged from 1 to 256 μg/ml. To demonstrate the cause-effect relationship between type of mutation at positions T2182C and A2223G and clarithromycin resistance, *in vitro* site-directed mutagenesis should be performed. The site-directed point mutations in the 23S rRNA gene introduced into clarithromycin-susceptible strain of *H.pylori* by natural transformation (42).

By site-directed mutagenesis, some authors found mutation A2142C, -G, or -T and A2143C, -G, or -T (41). However, they found that an apparent preferential mutation, A2142G or A2143G, exists, due to the fact that these mutants have the highest growth rates and more stable resistance as well as that the MICs for them are higher (41,42,45).

Several authors have used other methods to detect mutations related to *H.pylori* clarithromycin resistance. Stone et al. (116) proposed a PCR-oligonucleotide ligation assay for detection of clarithromycin resistance in *H.pylori*, and Pina et al. (131) used a test involving amplification and colormetric hybridization in liquid phase. PCR-RFLP has been widely used due to its simplicity and may be performed in a few hours (41).

The PCR-reverse hybridization assay system is an alternative simple and cost effective way of detecting resistance mutations and is highly suitable for testing large numbers of samples. Doorn et al. (143), compared to the conventional methods, the PCR-reverse hybridization detected additional mutants and appears to provide more accurate data about the presence of bacterial variants in the culture, especially when multiple strains are present. In contrast, direct sequence analysis of PCR products will often detect only the predominant sequence. Only if different mutants are present in approximately

similar concentrations are they detected by sequence analysis, whereas the reverse hybridization specifically detects small amounts of each mutant. Also, the reverse hybridization format permits the addition of specific probes in case additional relevants, even if they represent only a small proportion of a bacterial population, therefore, it could be particularly suitable to monitor the development of resistance during antibiotic therapy. The method can also be used directly in gastric biopsies, without the need for a bacterial culture.

CHAPTER VII

CONCLUSION

Of the 71 *H.pylori* isolates from 150 gastric biopsy, 40 isolates were susceptible to clarithromycin (MIC, 0.016 to 0.5 μg/ml), and the remaining 31 isolates were resistance to clarithromycin (MIC, $\geq$ 1 to 256 μg/ml). No differences in MICs were found for all the resistant strains when these were tested by E-test as well as by the agar dilution method.

All of these isolates were determined for point mutation by PCR-sequencing and reverse hybridization. Both methods gave concordant results. The mutation found in our resistant isolates were the transition A2143G, T2182C, and A2223G, but mutation at position 2142 was not found.

Resistance to clarithromycin is the main predictor of failure of eradication treatments including this compound. Since high resistant isolates (43.66%) was found in this study, it is necessary to determined drug resistance in *H.pylori*. To render the resistance detection process more effective, results must be available rapidly. Reverse hybridization provides a result within a few hours once the strain has been isolated, compared to 2 to 3 days when a standard phenotypic method is used.

The high sensitivity of the PCR- reverse hybridization method provides more accurate data, especially when multiple strains are present. Therefore, this method could facilitate further epidemiological and clinical studies.

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APPENDICES

APPENDIX I

Media, Solution and Identification procedures

Media

1. Brain heart infusion agar with 7% horse blood

Brain heart infusion	37	g/L
Bacto agar	13	g/L
Sheep blood	70	ml/L
Distilled water	1	L

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

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

2. Brain heart infusion agar with 7% horse blood and antibiotics

Brain heart infusion	37	g/L
Bacto agar	13	g/L
Sheep blood	70	ml/L
Vancomycin (300 μg/ml stock)	10	ml
Amphotericin B (200 μg/ml stock)	10	ml
Distilled water	1	L

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

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

3. Antibiotic solution preparation (stock solution)

Vancomycin, final concentration 3 μg/ml

- Prepare a stock solution of 300 μg/ml, dissolve 0.012 gm in 40 ml distilled water

Amphotericin B, final concentration 2 μg/ml

- Prepare a stock solution of 200 μg/ml, dissolve 0.008 gm in 40 ml distilled water

Note : Sterilize all antibiotic solutions by filtration through a 0.22 μm filter and store at -20 °C.

4. Brain heart infusion broth with yeast extract and horse serum

Brain heart infusion	37	g/L
Yeast extract	2.5	g/L
Horse serum	100	ml
Distilled water	900	ml

Sterilize by autoclaving at 121°C, 15 pounds/inch^2 pressure, for 15 minutes. Add horse serum after cooling base medium to 45°C to 50°C. Alternatively, the base medium can be filter sterilized by passage through a 0.45 or 0.22 μm filter system, then adding the horse serum aseptically. Mix and store at 4°C until used.

5. Mueller- Hinton agar containing 5% sheep blood (Susceptibility testing)

Mueller-Hinton agar	35	g/L
Sheep blood	50	ml/L
Distilled water	1	L

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

6. Transport medium with 20% glycerol (Biopsy specimens)

The transport medium is prepared by mixing 2 solutions :

SOLUTION A : FOR 200 ml

Casamino acids	2	g
Peptone	2	g
Yeast extract	0.4	g
Sodium chloride	1	g
Bacto agar	0.32	g

Add the ingredient to 200 ml of distilled water ; heat with stirring until the agar is dissolved. Sterilize by autoclaving at 121°C, 15 pounds/inch^2 pressure, for 15 minutes.

SOLUTION B : FOR 40 ml

L-Cysteine	0.04	g
Dextrose (glucose)	0.2	g

Dissolve cysteine in 20 ml of distilled water. Adjust to pH 7.0 with 0.5 N NaOH. Add the dextrose to the cysteine and bring to 40 ml. Sterilize by filtration (use a 0.22 μm filter). Mix solution A, 200 ml with solution B, 40 ml and 48 ml sterile glycerol. Aliquot into sterile screw cap tubes (1 mL/tube). Test the sterility of the cysteine medium by spreading 0.1 ml of the medium (3 tubes picked at random) onto the surface of an agar plate. Incubate the plate at 37°C for 24 hours. Store tubes in refrigerator at 4°C until used.

7. Brain heart infusion broth with yeast extract and horse serum (stock media)

Brain heart infusion	35	g
Yeast extract	1.5	g
Horse serum	50	ml
Glycerol	300	ml
Distilled water	700	ml

8. Urease reagent (culture identification)

Solution A : 2X Based Medium

Peptone	0.5	g
NaCl	2.5	g
KH_2PO_4	1.0	g

Dissolve in 250 ml distilled water, then autoclave at 15 pounds/inch^2 pressure, for 15 minutes.

Solution B : 2X Based Medium

Glucose	0.5	g
Urea	10	g
0.5% phenol red	1.6	ml

Dissolve in 250 ml distilled water, then sterilize by filtration through a 0.22 μm filter.

Mix equal volumes of solution A and solution B and aliquot in 10 ml screw capped tubes. Store at 4°C until used.

9. Sterile saline solution (suspending of bacterial inocula)

Sodium chloride	8.5	g/L
Distilled water	1	L

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

APPENDIX II

REAGENTS, MATERIALS AND INSTRUMENTS

A. REAGENTS

Absolute ethanol	(Merck, Germany)
Agarose (ultrapure)	(Biorad, U.S.A)
Brain heart infusion agar	(Oxoid, U.S.A.)
Brain heart infusion broth	(Oxoid, U.S.A.)
ECL detection reagent	(Amresco, U.S.A)
EDAC	(Sigma, U.S.A.)
EDTA	(Amresco, U.S.A)
Ethidium bromide	(Amresco, U.S.A)
Glacial acetic acid	(Merck, Germany)
Horse serum	(GibcoBRL, U.S.A.)
Mineral oil	(Sigma, U.S.A.)
Mueller-Hinton agar	(Bio-rad,U.S.A.)
NaCl	(Merck, Germany)
$NaHCO_3$	(Merck, Germany)
$Na_2HPO_4*2H_2O$	(Sigma, U.S.A.)
NaOH pellet	(Merck, Germany)
SDS	(Amresco,U.S.A.)
streptavidin-peroxidase conjugate	(Amresco, U.S.A)
Tris (ultrapure)	(Amresco, U.S.A)

B. MATERIALS

Anaerobic chamber	(BBL, U.S.A.)
Biodyne C membrane	(Amresco,U.S.A)

Hyperfilm ECL	(Amresco, U.S.A)
Dotblotter	(BBL, U.S.A.)
X-ray film	(Kodak, Japan)

C. INSTRUMENTS

Hybaid OmniGene thermal cycler	(Hybaid, England)
Water bath	(Memmert, U.S.A)
Perkin Elmer GeneAmp PCR system 9600	(Perkin Elmer, U.S.A)
ABI Prism™ 310 Automate sequencer	(Perkin Elmer, U.S.A)
Chemi Doc	(BIORAD, U.S.A.)
Incubator	(Forma Scientific, U.S.A.)
Microcentrifuge	(Eppendorf, U.S.A.)
Spectrophotometer	(BIORAD, U.S.A.)

APPENDIX III

REAGENTS AND PREPARATIONS

1. 0.5 M EDTA, pH 8.0

Disodium ethylene diamine tetraacetate.$2H_2O$	186.1	g
DDW	1,000	ml

Adjust pH to 8.0, Adjust volume to 1,000 ml

Store at room temperature for no longer than 1 year.

2. 1 M Tris-HCl, pH 8.0

Tris (ultrapure)	121.1	g
DDW	800.0	ml
Adjust to pH 8.0 by adding conc. HCl	42.0	ml

Sterilize by autoclaving

3. 50 x Tris-acetate buffer (TAE)

Tris (ultrapure)	242.0	g
Glacial acetic acid	57.1	g
0.5 M EDTA pH 8.0	100.0	ml

Adjust the volume to 1,000 ml with DDW

Sterilize by autoclaving

4. 5 M NaOH

NaOH	200	g

Adjust the volume to 1,000 ml with DDW

5. 20 x SSPE

0.2 M $Na_2HPO_4 * 2H_2O$	35.6	g
3.6 M NaCl	210.24	g
20 mM EDTA	7.4	g

Adjust to pH 7.4, Adjust volume to 1,000 ml, Sterilize by autolaving

Store at room temperature for no longer than one year.

6. 10 x TE buffer

Tris	12.11	g
0.5 M EDTA	20	ml

Adjust to pH 8.0 by adding conc. HCl, Adjust volume to 1,000 ml

Sterilize by autolaving

7. 10 % SDS

SDS	10	g
DDW	100	ml

Dissolve by heating at 65°C for 20 min., Do not autoclaving

8. 0.5 M $NaHCO_3$, pH 8.4

$NaHCO_3$	10.5	g
DDW	250	ml

Adjust to pH 8.4, Adjust volume to 250 ml with DDW

Store at room temperature for no longer than one year.

9. 16 % (w/v) EDAC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide)

EDAC	1.6	g
DDW	10	ml

Prepare fresh before use.

10. 2 X SSPE

20 X SSPE	10	ml
DDW	90	ml

Prepare fresh before use.

11. 0.1 M NaOH

NaOH	0.4	g
DDW	100	ml

Prepare fresh before use.

12. 20mM EDTA, pH 8.0

0.5 M NaOH (pH 8.0)	4	ml
DDW	96	ml

Store at room temperature for no longer than one year.

13. 2 X SSPE/0.1 % SDS

20 X SSPE	50	ml
10 % SDS	5	ml
DDW	445	ml

Prepare fresh before use.

14. 2 X SSPE/0.5 % SDS

20 X SSPE	50	ml
10 % SDS	25	ml
DDW	425	ml

Prepare fresh before use.

15. ECL detection reagent

ECL detection reagent 1	5	ml
ECL detection reagent 2	5	ml

Store at 4°C for no longer than six months.

BIOGRAPHY

Miss Somwai Leetranont was born on July 3, 1976 in Bangkok, Thailand. She graduated with bachelor degree of Veterinary Medicine in Mahanakorn University of Technology in 1999.