คุณลักษณะของยีน *rdxA*, *frxA*, *fdxA* และ *hefA* ในเชื้อ *Helicobacter pylori* ที่ดื้อยา metronidazole

นางสาวอรศิริ เชื้ออินทร์

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CHARACTERIZATION OF *RDXA*, *FRXA*, *FDXA* AND *HEFA* GENES IN METRONIDAZOLE-RESISTANT *HELICOBACTER PYLORI*

Miss Ornsiri Chueain

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Thesis Title	CHARACTERIZATION OF *RDXA*, *FRXA*, *FDXA* AND *HEFA* GENES IN METRONIDAZOLE-RESISTANT *HELICOBACTER PYLORI*
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อรศิริ เชื้ออินทร์ : คุณลักษณะของยีน *rdxA*, *frxA*, *fdxA* และ *hefA* ในเชื้อ *Helicobacter pylori* ที่ดื้อยา metronidazole(Characterization of *rdxA*, *frxA*, *fdxA* and *hefA* genes in metronidazole-resistant *Helicobacter pylori*) อ. ที่ปรึกษาวิทยานิพนธ์หลัก :อ. ดร. ธนิษฐา ฉัตรสุวรรณ 103 หน้า.

Metronidazole เป็นยาปฏิชีวนะที่ใช้ร่วมกับยาชนิดอื่นๆ ในการรักษาการติดเชื้อ *Helicobacter pylori* ซึ่งมักใช้การรักษาแบบ Triple therapy อย่างไรก็ตามมีรายงานการดื้อยา metronidazole เพิ่มสูงขึ้นสำหรับกลไกการดื้อยา metronidazole ในเชื้อ *H. pylori* ยังไม่ทราบที่แน่ชัด การดื้อยา metronidazole มีรายงานว่าเกิดจากกลายพันธุ์ของยีน *rdxA* ซึ่งสร้างเอนไซม์ oxygen-insensitive NADPH nitroreductase และการกลายพันธุ์ในยีนที่สร้างเอนไซม์ nitroreductase ชนิดอื่นๆ ได้แก่ ยีน fdxA และ ยีน frxA วัตถุประสงค์ในการศึกษาครั้งนี้เพื่อตรวจหาการกลายพันธุ์และการแสดงออกของยีน *rdxA*, *frxA* และ *fdxA* ใน *H. pylori* ที่ดื้อยา และเพื่อตรวจหาการแสดงออกของยีน *hefA* ในการขับยาออกจากเซลล์ เชื้อที่ใช้ในการศึกษาครั้งนี้เป็นเชื้อที่ดื้อยา metronidazole จำนวน 30 สายพันธุ์ และเชื้อที่ไวต่อยา metronidazole จำนวน 5 สายพันธุ์ ผลการศึกษาพบการกลายพันธุ์ของยีน *rdxA* ซึ่งทำให้มีการเปลี่ยนแปลงกรดอะมิโน 30 ตำแหน่ง และ 15 ตำแหน่งพบทั้งในเชื้อ *H. pylori* ที่ดื้อและไวต่อ metronidazole การกลายพันธุ์แบบ Frameshift mutation ซึ่งทำให้เกิด stop codon พบในเชื้อดื้อยา metronidazole จำนวน 10 สายพันธุ์ สำหรับใน FdxA พบการเปลี่ยนแปลงของกรดอะมิโนน้อยมาก โดยพบ amiono acid substitution 1 ตำแหน่งในเชื้อดื้อยา metronidazole 1 สายพันธุ์ และ amino acid deletion ที่ตำแหน่ง 47 เนื่องจากการหายไปของนิวคลีโอไทด์ TGA ในเชื้อดื้อยา 6 สายพันธุ์ และไม่พบการเปลี่ยนแปลงของลำดับกรดอะมิโนของ FdxA ในเชื้อที่ไวต่อยา ใน FrxA พบว่าเชื้อดื้อยา 15 สายพันธุ์มีการกลายพันธุ์แบบ Frameshift mutation ซึ่งทำให้เกิด stop codon และพบ amino acid substitution ในเชื้อที่ไวต่อยา 4 สายพันธุ์ จากการศึกษาการกลายพันธุ์ของ nitroreductases ในเชื้อดื้อยาทั้งหมด 30 สายพันธุ์ พบการกลายพันธุ์ร่วมกัน 3 ยีนใน 7 สายพันธุ์ (23.33%) และทุกสายพันธุ์จะพบการกลายพันธุ์อย่างน้อยสองยีนเป็นอย่างน้อย (100%) การศึกษาการแสดงออกของยีนที่สร้าง nitroreductase และ HefA ไม่พบ mRNA expression โดยวิธี RT-PCR ทั้งในเชื้อที่ดื้อและไวต่อยา metronidazole ซึ่งในการศึกษาต่อไปจะนำวิธีที่มีความไวกว่าได้แก่วิธี Real-time RT-PCR มาใช้ในการศึกษา ส่วนการศึกษากลไกการขับยาออกจากเซลล์ HefA ไม่พบการลดลงของ MIC ของยา metronidazole เมื่อใส่ CCCP ซึ่งเป็นตัวยับยั้ง efflux pump การศึกษาครั้งนี้พบว่าการกลายพันธุ์ในยีนที่สร้าง nitroreductases ได้แก่ *rdxA, fdxA* และ *frxA* มีความสัมพันธ์กับการดื้อยา metronidazole

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต .....

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ORNSIRI CHUEAIN: CHARACTERIZATION OF *RDXA*, *FRXA*, *FDXA* AND *HEFA* GENES IN METRONIDAZOLE-RESISTANT *HELICOBACTER PYLORI*.

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Metronidazole is a bactericidal antibiotic that one component of the triple drug therapy that commonly used for treating *Helicobacter pylori* infection. However, metronidazole resistance rate has been incleasingly report. The mechanism of metronidazole resistance in *H. pylori* is still unclear. The acquisition of metronidazole resistance is reported to be associated with mutational inactivation of the *rdxA* gene, which encodes an oxygen-insensitive NADPH nitroreductase. Recent evidence has suggested that mutations in other nitroreductase-encoding genes including *frxA* and *fdxA* may contribute to the resistance phenotype. The aims of this study are to determine mutations and expression of *rdxA*, *frxA* and *fdxA* in metronidazole-resistant *H. pylori* and to determine expression of *hefA* gene in metronidazole-resistant *H. pylori*. There were 30 metronidazole-resistant and 5 metronidazole-susceptible isolates included in this study. The results showed that 30 amino acid substitutions were found in RdxA. There were 15 amino acid substitution found in both susceptible and resistant isolates. Frameshift mutation leading to premutured stop codon was found in 10 metronidazole-resistant isolates. Few alterations in amino acid sequences of FdxA were observed. One amino acid substitution was found in metronidazole-resistant isolate,828, and amino acid deletion by nucleotide TGA deletion was found in 6 metronidazole-resistant isolates. No mutation in FdxA amino acid sequences was found in metronidazole-susceptible isolates. In FrxA, 15 metronidzole-resistant isolates had the frameshift mutation, leading to stop codon, and amino acid substitutions were present in 4 metronidazole-susceptible isolates. The results showed that mutations in RdxA together with FdxA and FrxA were observed in 7(23.33%) and 30(100%) of 30 metronidazole-resistant isolates, respectively, and combination of 3 gene mutations was found in 7(23.33%) metronidazole-resistant isolates, and all of metronidazole-resistant isolates were found at least two mutation in these nitroreductase genes (100%). For study of the efflux pump mechanism (HefA), no detection the decreasing of MIC level of metronidazole with CCCP which the efflux pump inhibitor. RT-PCR could not detect mRNA expression of nitroreductase genes and HefA in both susceptible and resistant isolates. A sensitive method, Real-time RT-PCR, is considered to be used for further investigation. Our results demonstrated that mutations in nitro reductase genes including *rdxA*, *frxA* and *fdxA* were associated with metronidazole resistance in *H. pylori*

Field of Study : Medical Microbiology Student's Signature .....

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LIST OF ABBREVIATIONS

A	adenine
bp	base pair
C	cytosine
CO_2	carbon dioxide
^oC	degree celsius
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
DDW	double distilled water
dGTP	deoxyguanosine 5'-triphosphate
DNA	deoxynucleic acid
dNTPs	deoxynucleotide-triphosphate
dTTP	deoxythymidine 5'-triphosphate
DW	distilled water
et al.	*et alii*
E-test	epsilometer test
g	gram
G	guanine
HCl	hydrochloric acid
hr	hour
i.e.	id test
M	molar
mg	milligram
$MgCl_2$	magnesium chloride
MIC	minimum inhibitory concentration
min	minute (S)

ml	milliliter
mM	millimolar
mmol	millimole
MTZ	metronidazole
NaCl	sodium chloride
Na_2HPO_4	sodium phosphate dibasic, anhydrous
NaOH	sodium hydroxide
CLSI	Institute Clinical Laboratory Standards
PCR	polymerase chain reaction
Pfu	*Pyrococcus furiosus*
*P*mol	picomol
sec	second
T	thymine
Tris	Tris-(hydroxymethyl)-aminoethane
U	unit
µg	microgram
µl	microliter
µM	micromolar
UV	ultraviolet
V	volt

CHAPTER I

INTRODUCTION

Helicobacter pylori is a curved, microaerophilic, Gram-negative bacterium that was first isolated from a stomach biopsy by Warren and Marshall in 1982(1). It was initially named *Campylobacter pyloridis* because of phenotypic characteristic similar to those of *Campylobacter*. Then, it was placed in its own genus, *Helicobacter* and known as *H. pylori* (2). *H. pylori* is an important human pathogen that colonizes the stomach of about half of the world‟s population and the prevalence varies widely according to different geographic area, age, race, and socioeconomic status, sanitation and/or living conditions, and correlates with a low socioeconomic status during childhood (3). *H. pylori* has now been accepted as the causative agent of several gastroduodenal disorders, ranging from chronic active gastritis and peptic ulcer disease to gastric cancer (2). Triple drug regimens are highly effective for the treatment of *H. pylori* infection. However bacterial resistance to one of the most effective antibiotics, metronidazole, is a serious and increasing problem (4). In developing countries the metronidazole resistance is high, probably as a consequence of the frequent use of metronidazole for parasitic infections (5).

The activity of metronidazole in *H. pylori* is dependent on reduction of its nitro moiety to highly reactive compounds that cause DNA strand breakage(6). The mechanism of metronidazole resistance in *H. pylori* is still unclear. The acquisition of resistance was reported to be associated with mutational inactivation of the *rdxA* gene, which encodes an oxygen-insensitive NADPH nitroreductase. Many studies have subsequently shown that the majority of metronidazole-resistant *H. pylori* strains contained various mutations within the *rdxA* gene, including frameshift mutation, leading to stop codons, nucleotide insertion and deletion, resulting in amino acid substitution, and promoter alterations (7, 8). However, it appears that the mechanism of metronidazole resistance in *H. pylori* is complex, as indications for the involvement of other nitroreductase-encoding genes associated. Mutational changes of the *frxA* (NADPH flavin oxidoreductase) and *fdxA* (ferredoxin-like protein) gene may contribute to metronidazole resistance. Recent evidence has suggested that inactivation of other nitroreductase-encoding genes including *frxA* and *fdxA* may

contribute to the resistance phenotype (4). It was reported that susceptibility of *H. pylori* to metronidazole was attributed to the activity of an oxygen-insensitive NADPH-dependent nitroreductase (RdxA). Possible mechanisms of intrinsic drug resistance involve decreased drug uptake or increased drug efflux. The resistance-nodulation-division (RND) family of efflux systems is the one of five family of efflux system that is widespread in gram-negative bacteria. The RND family of efflux systems has three components: inner membrane efflux proteins (IEPs) which act with the other two components, a periplasmic efflux protein (PEP) which facilitates interaction with the other two components, and an outer membrane efflux protein (OEP) which is the TolC (the outer membrane efflux protein in *Escherichia coli*) or a TolC homolog. It is a multidrug efflux that can pump out an extremely wide range of substrates, including antibiotics, chemotherapeutic agents, metabolic inhibitors such as cerulenin, dyes, detergents, and solvents. Previous study in *H. pylori* demonstrated the presence of three putative RND efflux systems, including *hefABC*, *hefDEF*, and *hefGHI* (9). Of the these efflux systems, only HefABC showed homology to the RND efflux systems which involved in multidrug resistance in other bacteria. This suggests that HefA efflux pump, TolC homolog may play a role in metronidazole resistance mechanism (10, 11). There are only few studies on the mechanisms of metronidazole resistance in *H. pylori* in Thailand. In this study, we investigated metronidazole resistance mechanisms in *H. pylori* by examining nitroreductase gene mutations in *rdxA* and other oxygen-sensitive nitroreductase-encoding genes including *frxA* and *fdxA*. The mRNA expression levels and nitroreductase activity were also evaluated. We also investigated the efflux pump mechanism by using CCCP and determined the HefA expression level.

CHAPTER II

OBJECTIVES

I. To determine mutations and expression of *rdxA, frxA* and *fdxA* in metronidazole-resistant *H. pylori*

II. To determine expression of *hefA* gene in metronidazole-resistant *H. pylori*

CHAPTER III

LITERATURE REVIEW

1. BACTERIOLOGY

In 1982, Warren and Marshall were the first to isolated *Helicobacter pylori* from cultivaton of the gastric biopsy samples (1) *H. pylori* was classified first as *Campylobacter*-like organism and *Campylobacter pyloridis* , then renamed *C. pylori* because the similarlity of characteristics later, it was placed in its own genus, *Helicobacter* and known as *Helicobacter pylori* (12). *H. pylori* is a Gram-negative, spiral rod bacterium. In biopsy specimens, it is 2.5 to 5.0 μm in length and 0.5 to 1.0 μm in width and contain 4 to 6 unipolar sheathed flagella approximately 30 μm long and 2.5 nm wide. In fresh medium, *H. pylori* has spiral rod shaped, but prolonged culture it may change into a coccoid form typically predominate. A *H. pylori* is fastidious and slow-growing bacterium, it takes 3 to 5 days and for maximum of 7 to 10 days at 35 to 37 °C for visible colonies to form in rich culture medium with supplement with blood & serum under a microaerophilic atmosphere. The colony morphology that identified by translucent, small, circular, smooth colonies varying in

size from barely detectable with the naked eyes to approximately 3 mm. *H. pylori* can produce certain enzymes such as cytochrome oxidase, catalase, and urease(13).

2. EPIDEMIOLOGY

H. pylori is commonly found in the stomach of humans. It is present in approximately one-half of the world's population and occurs high prevalence worldwide. In industrialized countries, 25 to 50% of the middle-aged adults are infected with *H. pylori*. The data from developed countries also suggest that most infections are acquired in childhood. In developing countries, it has been reported that 70 to 90% of the population carried *H. pylori*. Almost all of these acquired the infection before the age of 10 years. The epidemiology of *H. pylori* infection varied widely by geographic area, age, race, and socioeconomic status sanitation and/or living conditions (2, 3) The epidemiology of *H. pylori* infection in developing countries, such as India, Saudi Arabia or Vietnam, is characterised by a rapid rate of acquisition of the infection such that approximately 80% of the population is infected by the age of 20 because the disease is most often acquired in childhood(Figure 1). In developing countries the prevalence of infection peaks in the 20 to 30 year old age group. The prevalence of the infection varies between subpopulations within the same country, especially in relation to age. the patterns in developed countries such as the United Kingdom, Australia and France that also show an increasing prevalence with age.

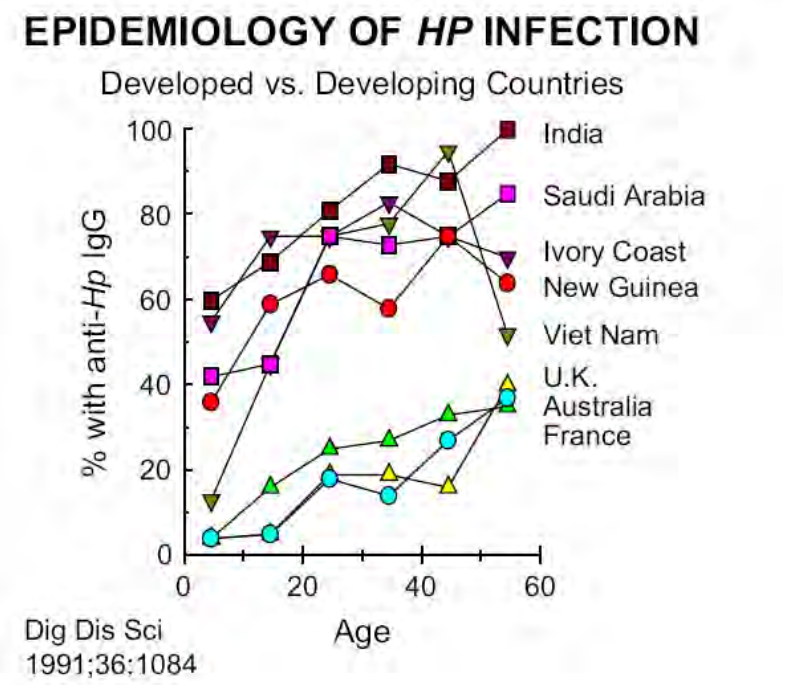

Figure 1. Comparison of the prevalence of *H. pylori* infection in industrialised and non-industrialised countries, as indicated by the presence of serum IgG antibodies to *H. pylori* antigens (14).

3. PATHOGENESIS

Colonization with *H. pylori* is the common cause of inflammation in the gastric mucosa and is strongly linked to the development of chronic active gastritis, duodenal ulceration and peptic ulcer disease, and strongly associated with an increased risk of developing gastric cancer and gastric lymphoma. The discovery of *H. pylori* infection is now well accepted that the main cause of most stomach diseases and has significantly changed the management of gastroduodenal diseases. Furtheremore, It has been considered a risk factor for the development of gastric adenocacinoma and mucosa-associated lymphoid tissue (MALT) lymphoma (1, 2, 12, 15). Figure 2 shows the schematic representation of the natural history of *H. pylori* infection (5).

Transmission of *Helicobacter pylori* is thought to occur mainly during childhood, and predominantly within families. This bacterium travels from the stomach of one person to that of another by three routes. The first and least common route is iatrogenic, in which tubes, endoscopes, or specimens that exposed with the gastric mucosa from one person are introduced to another person. Secondly, fecal-oral route are believed to be the primary means of transmission and appears to be the most important route of transmission. Although *H. pylori* has been isolated from the feces of young children infected with the organism, the bacterium can also be transmitted through exposure to contaminated food or water. Thirdly, oral to oral transmission has been identified in the case of African women who premasticate food given to their infants (2, 5).

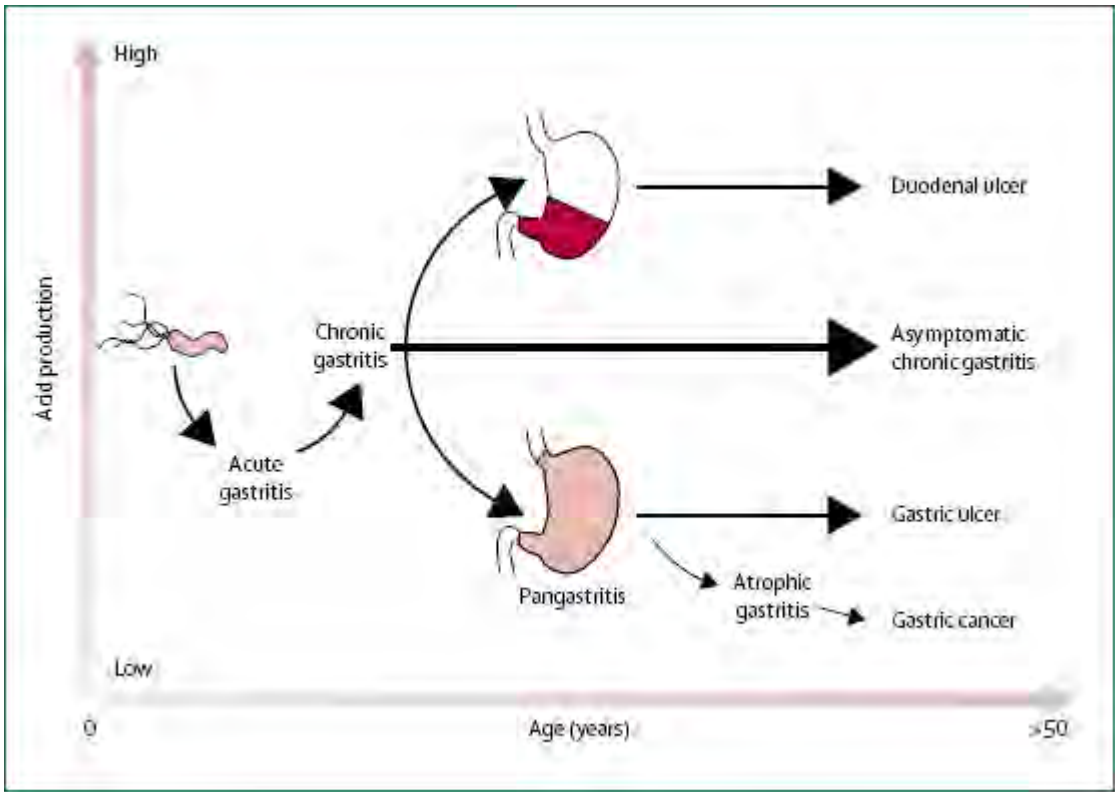

Figure 2. Schematic representation of the natural history of *H pylori* infection (5)

Acquisition of *H pylori* usually occurs during childhood. Once acquired and left untreated, the infection persists for life. After the acute phase, most *H pylori*-positive patients develop a chronic gastritis without symptoms. In some patients, more severe manifestations will develop later in life. A normal or high acid secretion predisposes to duodenal ulcers, whereas a low acid secretion predisposes to gastric ulcers and gastric cancer.

3.1 VIRULENCE FACTOR

H. pylori has virulence factors, which allow its survival in the human stomach. The vacuolating cytotoxin (VacA) and the cytotoxin associated antigen (CagA) are major bacterial factors involved in modulating the host.

Cytotoxin associated gene *A (cagA)* and the *cag* pathogenicity island

CagA was first described as a protein which was expressed more commonly by toxigenic than non-toxigenic strains. It is a highly immunogenic protein which translocated into the intracellular region of host cells through the type IV secretion system, which is encoded by the *cag* pathogenicity island that trigger inflammation and cell proliferation (2, 16). Both may contribute to the harmful effects of *H. pylori*.

VacA vacuolating cytotoxin

Approximately 50% of all *H. pylori* strains secrete VacA, a highly immunogenic 95-kDa protein that induces massive vacuolization in epithelial cells *in vitro* (17, 18). The VacA protein plays an important role in the pathogenesis of both peptic ulceration and gastric cancer. In addition to inducing vacuolation, VacA can induce multiple cellular activities, including membrane-channel formation, cytochrome *c* release from mitochondria leading to apoptosis, and binding to cell-membrane receptors followed by initiation of a proinflammatory response. Although VacA is not essential for *in vitro* growth of *H. pylori*, it was reported to significantly contribute to murine gastric colonization by *H. pylori* (2, 19).

4. DIAGNOSIS OF *H. PYLORI* INFECTIONS

Infection of *H. pylori* can be diagnosed by a variety of invasive and non-invasive tests (20). Invasive tests are based on gastric samples, usually mucosal biopsies, which can be screened by rapid urease test, histology, or culture. Noninvasive tests require alternative clinical specimens, such as blood, breath, faeces, urine, or saliva. (2, 17)

4.1 Invasive tests

4.1.1 Histology

Histologic assessment has traditionally been the gold standard method for diagnosing *H. pylori* infection. *H. pylori* can be visualized at high magnification with conventional hematoxylin and eosin (H & E) stained sections. supplementary stains (such as Giemsa, Genta, Gimenez, Warthin-Starry silver, Creosyl violet) are needed to detect low levels of infection and to show the characteristic morphology of *H. pylori*. An important advantage of histology is that, in addition to the historical record provided, sections from biopsies (or even additional sections) can be examined at any time, and that gastritis, atrophy, or intestinal metaplasia can also be assessed (2).

4.1.2 Culture

Because *H. pylori* is difficult to grow on culture media, and has a risk of overgrowth or contamination, culture is the least sensitive method of detection. Although costly, time-consuming, and labor intensive, culture has a role in antibiotic susceptibility studies and studies of growth factors and metabolism. Although only a few centres routinely offer microbiological isolation of *H. pylori*, culture and antibiotic sensitivity testing in the places where multiple resistant strains have been reported, may become a requirement for patients with persistent infection after initial or repeated treatment failure.

4.1.3 Urease tests

Rapid urease testing takes advantage of the fact that *H. pylori* is a urease-producing organism (13). This method are quick and simple for detecting *H. pylori* infection but indicate only the presence or absence of infection. The CLO test are of similar sensitivity and specificity. However, the sensitivity of urease tests is often higher than that of other biopsy based methods because the entire biopsy specimen is placed in the media, thereby avoiding the additional sampling or processing error associated with histology or culture. The sensitivity of biopsy urease tests seems to be much lower (~60%) in patients with upper gastrointestinal bleeding, but this can be improved by placing multiple biopsy samples into the same test vials.

4.1.4 Polymerase Chain Reaction (PCR)

PCR technique is highly sensitive and specific. The PCR consists of amplifying DNA sequences Specific for *H. pylori*. The factor that effect on the accuracy of this test are the used primers for amplify the PCR products, target DNA, and bacterial density. PCR tests for *H. pylori* have been described for a number of genomic targets such as urease gene and virulence genes (such as *cagA* and *vacA*) (13).

4.2 Non-invasive tests

4.2.1 Serology

In response to *H. pylori* infection, the immune system typically rises a response through production of immunoglobulins to organism-specific antigens Circulating IgG antibodies to *H. pylori* can be detected by enzyme-linked immunosorbent assay (ELISA) antibody or latex agglutination tests. These antibodies can be detected in serum or whole-blood samples. Serologic tests are generally simple, fast, reproducible, inexpensive, and can be done on stored samples (13).

However this method cannot be used to determine *H. pylori* eradication or to measure reinfection rates because antibody titres fall slowly after successful eradication. Serologic tests may be useful in identifying certain strains of more virulent *H. pylori* by detecting antibodies to virulence factors associated with more severe diseases and complicated ulcers, gastric cancer, and lymphoma.

4.2.2 Urea breath test

A urea breath test is based on the urease activity of *H. pylori* to detect the presence of active infection. The ^{13}C-urea breath test the principle that a solution of urea labelled with carbon-13 will be rapidly hydrolysed by the urease enzyme of *H. pylori*. The resulting CO_2 is absorbed across the gastric mucosa through the systemic circulation, excreted as $13CO_2$ in the expired breath. The 13C-urea breath test detects current infection and it has the advantage of being nonradioactive and thus safer for patient. It can be used as a screening test for *H pylori*, to assess eradication and to detect infection in children. The similar but radioactive 14C-urea breath test cannot be performed in primary care (2).

4.2.3 Stool antigen test

Stool antigen test is a relatively new methodology that uses a simple sandwich ELISA to detect the presence of *H. pylori* antigen in stool specimens. A cost effective and reliable means of diagnosing active infection and confirming cure. This test has a sensitivity and specificity comparable to those of other noninvasive tests and the technique has the potential to be developed as a near patient test. The main advantage of the test, however, is in large scale epidemiological studies of acquisition of *H. pylori* in children (2).

Table 1. Diagnosis of *H. pylori* infection(17)

Diagnostic method	Sensitivity	specificity[a]	Typical application	Remarks
Invasive method				
Histology	96.6%	100%	"Gold standard" in routine hospital diagnostics	Requires expert pathologist; also provides histological data on inflammation and atrophy
Culture biopsy	98.3%	100%	Alternative gold standard	Allows for testing of antimicrobial sensitivity; requires specific microbiological expertise
Rapid urease (CLO) test	96%	90%	Cost-effective and rapid test	Requires an additional test for confirmation of *H. pylori* infection
Noninvasive methods				
Urea breath test	95%	90%	Alternative gold standard	Very useful, reliable test to evaluate success of eradication treatment of *H. pylori*; limited availability due to requirement of expensive equipment
Fecal antigen test	96%	96%	Not widely used yet	Simple test but may not be reliable for evaluation of success of eradication treatment of *H. pylori*
Serology	90-97%,	50-96%	Mainly used for epidemiological studies	Insufficient reliability for routine screening; cannot prove ongoing infection due to immunological memory
PCR (of tissue or fluid samples)	100%	100%		Rapid determination of some phenotypic (antibiotic

[a] Global range, depending on regional variations and subjects

5. TREATMENT OF *H PYLORI* INFECTIONS

Once *H. pylori* is detected in patients with a peptic ulcer and other *H. pylori*-associated disorders, the normal procedure is to eradicate it and allow the ulcer to heal with antibiotics. Although *H. pylori* is sensitive to a wide range of antibiotics in vitro, they all fail as monotherapy *in vivo* (21). The most common used antimicrobial drugs for treatment of *H. pylori* are metronidazole, clarithromycin, amoxicillin and tetracycline (Table 2). Other antibiotics such as fluoroquinolones, nitrofurans, and rifamycins are occasionally used as alternatives (22, 23). Triple therapy, including of one or more antibiotics in combination with an acid-suppressive drug and/or a proton pump inhibitor. The use of proton pump inhibitors (PPI), such as omeprazole and lansoprazole were shown to be effective (24, 25). Acid-suppressive drugs primarily increasing the pH in the gastric mucosa, and increase the activity of the antibiotics. An additional beneficial effect of acid-suppressive drugs is that they decrease the severity of side effects of a given regimen. The acid-suppressive drugs that commonly used are bismuth subcitrate, bismuth subsalicylate and other bismuth compound. Inhibition of protein, ATP, and cell wall synthesis were the effect of bismuth salt on *H. pylori*. Triple therapies consisting of two antibiotics and a PPI or ranitidine bismuth citrate for 7-10 days are now mostly recommended. There seems to be preference for combination therapy that includes amoxicillin and clarithromycin. The first-line therapy is not successful that usually related to insufficient patient and/or development of antibiotic resistance. Standard third-line therapies are lacking due to a limited choice of antibiotics and the need to individualized treatment depending on which therapies previously failed. Most patients who remain *H. pylori*-positive after two sequential courses of eradication treatment have been infected with an *H. pylori* strain that is resistant to one or more of the previously used antibiotics. To select an appropriate third-line treatment, endoscopy followed by bacterial culture and antibiotic susceptibility testing is advisable. An overview of the commonly used triple- and quadruple therapies is given in Table 3 .

Metronidazole

Metronidazole are commonly agents in the treatment of several protozoa and anaerobic bacterial infections. Metronidazole are metabolized by *H. pylori* by several nitroreductases. The mode of action of metronidazole, it is administered as a prodrug that needs to be activated within the target cell by one or two electron reduction processes. This reduction leads to the formation of nitro-anion radicals and metronidazole intermediates that cause lethal damage to subcellular structures and DNA (4, 6, 26, 27) .

Macrolides

Clarithromycin is a bacteriostatic antibiotic that belongs to the group of macrolides that bind reversibly to the peptidyl transferase loop of domain V of the 23S ribosomal RNA (rRNA) molecule. This binding interferes with protein elongation, and thus effectively blocks bacterial protein synthesis. The antibacterial activity of clarithromycin is much the same as that of other macrolides, but clarithromycin is better absorbed in the gastric mucus layer and is more acid-stable (28).

Penicillins

Amoxicillin is a bactericidal antibiotic that belongs to thepenicillin group of drugs. The drug binds to penicillinbinding proteins (PBPs) and interferes with bacterial cell wall synthesis, resulting in lysis of replicating bacteria. The antibacterial activity of amoxicillin is much the same as that of other penicillins, but amoxicillin is better released in the gastric juice, and displays increased stability in acidic conditions compared with other penicillins (29).

Tetracycline

Tetracycline is a bacteriostatic antibiotic that binds to the 16S rRNA, that effect on interfering with the attachment of aminoacyl-tRNA to the ribosome, resulting in inhibition of protein synthesis and bacterial growth (30).

Fluoroquinolones

Fluoroquinolones are bactericidal antibiotics that exert their antimicrobial activity by inhibition of DNA gyrase. This enzyme is a tetramer that consists of two A subunits and two B subunits, encoded by the *gyrA* and *gyrB* genes, respectively (31, 32).

Nitrofurans

Furazolidone and nitrofurantoin are nitroheterocyclic and nitroaromatic compounds are bactericidal antibiotics that share similarities with metronidazole both in their structures and modes of action (33).

Rifamycin

Rifabutin and several other derivates of rifampin are bactericidal antibiotics The mode of action that bind to the β-subunit of DNA-dependent RNA polymerase. There was the effect to inhibition of transcription (34).

Table 2. Mode of action, resistance mechanisms, and prevalence of resistance among antimicrobials used for treatment of *H. pylori* infection(5)

Antimicrobial	Commonly used compound	Resistance rates*	Mode of action	Mechanism of resistance
Nitroimidazoles	Metronidazole, tinidazole	20–95%	Reduction of prodrug by nitroreductases leads to formation of nitro-anion radicals and imidazole intermediates and subsequent DNA damage	Absence of imidazole reduction caused by reduced or abolished activity of electron transport proteins (eg, RdxA, FrxA, FdxB)
Macrolides	Clarithromycin, erythromycin	0–50%	Binds 23S rRNA ribosomal subunit, resulting in inhibition of protein synthesis	Point mutations in 23S rRNA genes
Penicillins	Amoxicillin	0–30%	Binding of beta-lactam antibiotic to penicillin-binding proteins (PBP) inhibits cell division	Decreased binding of amoxicillin to PBP D (tolerance) or PBP1A (resistance caused by point mutation in the *pbp*1A gene), and reduced membrane permeability (resistance)
Tetracyclines	Tetracycline	0–10%	Binding to ribosome prevents association with aminoacyl-tRNA and subsequent protein synthesis	Point mutations in 16S rRNA genes and reduced membrane permeability
Fluoroquinolones	Ciprofloxacin, moxifloxacin, levofloxacin	0–20%	Inhibition of DNA gyrase and topoisomerases, interfering with DNA replication	Point mutations in the DNA gyrase gene, *gyrA*
Rifamycins	Rifabutin	0–2%	Binding to RNA polymerase, resulting in transcription inhibition	Point mutations in the RNA polymerase gene, *rpoB*
Nitrofurans	Furazolidone	0–5%	Reduction of prodrug by nitroreductases, leads to formation of nitro anion radicals and subsequent DNA damage	Unknown
Proton pump inhibitor	Omeprazole, lansoprazole, pantoprazole	Not reported	Inhibits the proton motive force of the bacterium, and destabilises its site of colonisation in the stomach	Unknown
Bismuth	Bismuth subcitrate, bismuth subsalicylate, ranitidine bismuth citrate	Not reported	Inhibits protein, ATP, and cell membrane synthesis	Unknown

*Prevalence of antimicrobial resistance in *H pylori* shows regional variation both within and between countries. In industrialised countries, the prevalence of resistance is lower than in developing countries.

Table 3. Current guidelines used for treatment of *H pylori* infections, based on the guidelines of the Maastricht 2-2000 Consensus, the National Institute for Clinical Excellence (NICE), and the European *Helicobacter pylori* Study Group (EHPSG)

Regimen*	Dose	Duration (days)
Triple therapy		
Ranitidine bismuth citrate, clarithromycin, and amoxicillin	400 mg, 500 mg, and 1 g, all twice daily	7–14
PPI, clarithromycin, and amoxicillin†	20–40 mg, 500 mg, and 1 g, all twice daily	7–14
PPI, clarithromycin, and metronidazole	20–40 mg, 500 mg, and 500 mg, all twice daily	7–14
PPI, amoxicillin, and metronidazole	20–40 mg, 1 g, and 500 mg, all twice daily	7–14
Quadruple therapy		
PPI, bismuth, metronidazole, and tetracycline	20–40 mg twice daily, 120 mg four times daily, 500 mg three times daily, and 500 mg four times daily	7–10‡

PPI=proton pump inhibitor. *Metronidazole can be replaced by tinidazole. †Therapy approved by the Food and Drug Administration (FDA). ‡Antibiotics are given for 4–7 days; PPI usually started 3 days earlier.

***Table 2*: Current guidelines used for treatment of *H pylori* infections, based on the guidelines of the Maastricht 2-2000 Consensus, the National Institute for Clinical Excellence (NICE), and the European *Helicobacter pylori* Study Group (EHPSG)**

6. ANTIBIOTIC RESISTANCE MECHANISMS

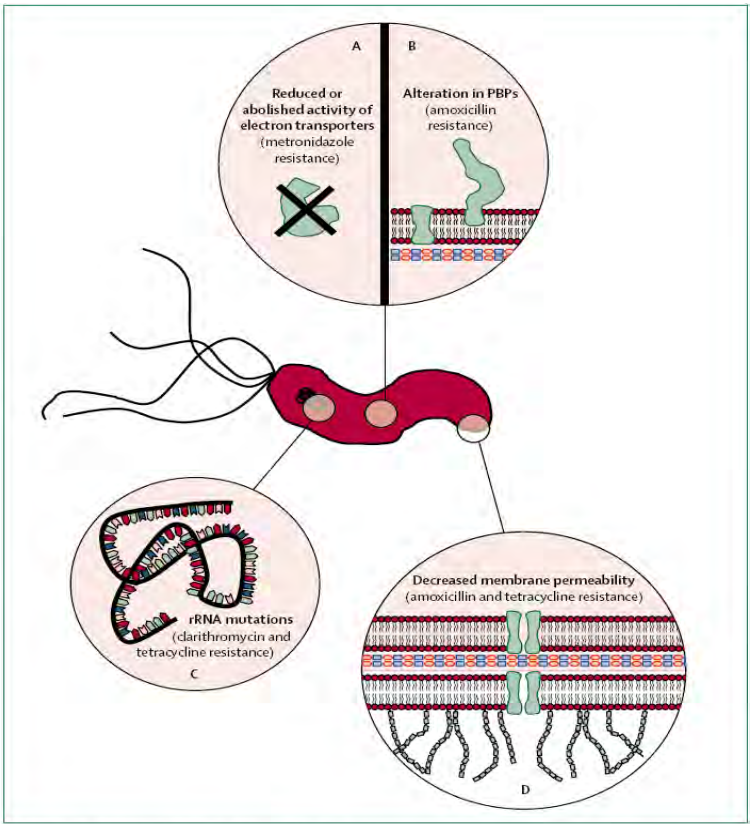

Figure 3. Mechanisms of antibiotic resistance in *H. pylori* (A) metronidazole, (B) amoxicillin, (C) clarithromycin and tetracycline, and (D) amoxicillin and tetracycline. (A) Reduced or abolished activity of electron transport proteins eg. RdxA, FrxA, or FdxB. (B) Alteration in the penicillin-binding proteins PBP-D and PBP1A. (C) Point mutations in the rRNA genes for 16S and 23S rRNA. (D) Decreased membrane permeability (5).

7. METRONIDAZOLE RESISTANCE

Resistance to nitroimidazoles is the most common form of antimicrobial resistance in *H. pylori*. The presence of nitroimidazole resistance is related to the previous use of this drug. The prevalence of resistance is rising and nowadays 10±50% of the isolates are resistant. In industrialised countries, about 35% of the *H. pylori* strains are resistant to nitroimidazoles (minimum inhibitory concentration [MIC] ≥8 µg/mL; susceptibility breakpoint).

All *H. pylori* isolates were susceptible to amoxicillin [MIC for 90% of the isolates (MIC90) <0.016 µg/mL], and only one tetracycline-resistant isolate was observed (MIC = 1.5 µg/mL). The overall prevalence of clarithromycin resistance was 8% during the 9-year study period, ranging from 0% (in 2000) to 16% (in 2003). The increase in clarithromycin resistance did not reach significance in the study period. Levofloxacin resistance varied between 0% and 12% (overall 7%), and metronidazole resistance varied between 29% and 59% (overall 41%).

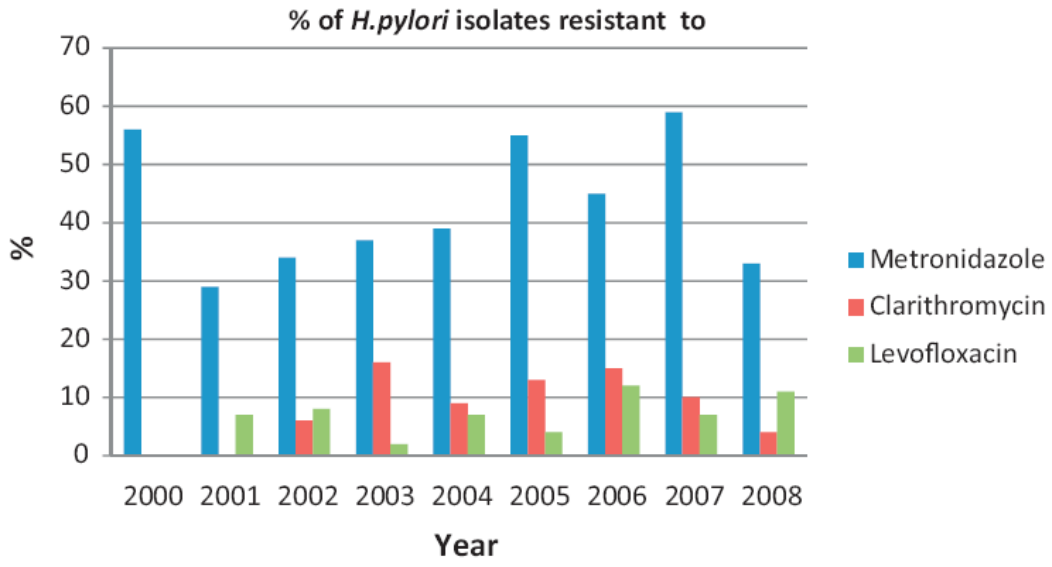

Figure 4. Antimicrobial resistance of *Helicobacter pylori* isolates during the study period (35).

8. METRONIDAZOLE RESISTANCE MECHANISMS

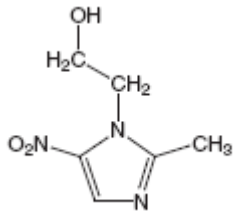

Figure 5. Structure of metronidazole ring

Metronidazole are bactericidal antibiotics that belong to the nitroimidazole group of drugs used for treatment of many infections caused by anaerobic bacteria, protozoa and some microaerophilic bacteria. After it has entered to the target cell by passive diffusion, metronidazole are given as a prodrug that needs to be activated intracellularly and become effective by one or two electron transfer processes (6, 26, 36). Metabolism of metronidazole occurs by a reduction step in which the drug is the electron acceptor. This reduction leads to the formation of nitro-anion radicals and other compounds, such as nitroso- and hydroxylamine compounds that cause lethal damage to macromolecules, subcellular structures and DNA, and subsequently results in the death of the bacterium. For the reduction step metabolized by several nitroreductases that are present in this bacterium, including ferredoxin (FdxA), flavodoxin (FldA), ferredoxin-like protein (FdxB), NAD(P)H flavin nitroreductase (FrxA), 2-oxoglutarate oxidoreductase (OorD), pyruvate:ferredoxin oxidoreductase (PorD), and oxygen-insensitive NAD(P)H nitroreductase (RdxA). In *H. pylori* it has been suggested that reduction of metronidazole is mainly mediated by an oxygen-insensitive NADPH nitroreductase encoded by the *rdxA* gene, but recently it has been shown that other nitroreductases such as flavin-oxidoreductase(FrxA) also participates in the reduction of metronidazole.

In the case of the reduction of the metronidazole that is metabolized by an oxygen-insensitive nitroreductase subsequently resulting in a nitrosoderivate by the simultaneous transfer of two electrons. This nitroso-derivate cannot be re-oxidized by molecular oxygen due to the chemical nature of the two-electron transfer step catalyzed by this enzyme. The nitroreductase is, therefore, called „oxygen-insensitive". This highly toxic nitrosoderivate causes DNA damage such as DNA double strand breakage and subsequent killing of the bacterium (26) (Figure 6).

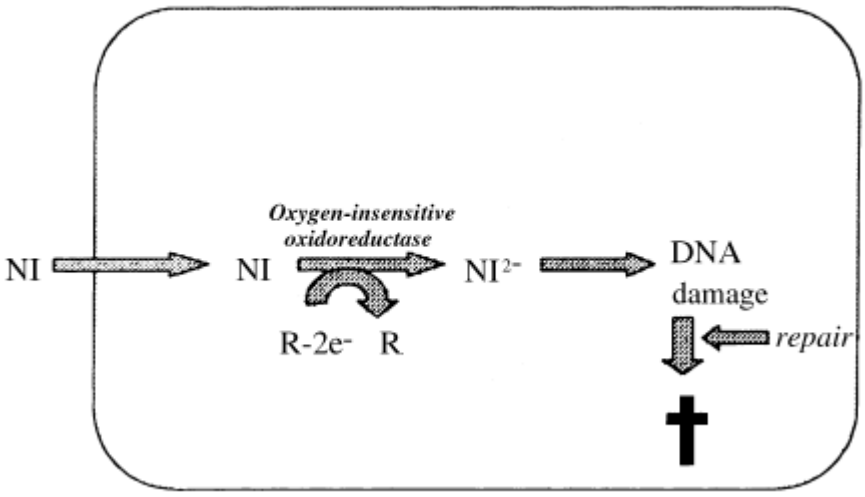

Figure 6. Metabolism of a nitroimidazole (NI) by an oxygen-insensitive nitroreductase

In contrast, nitroimidazole is reduced by other nitroreductases by a one-electron transfer step to a toxic free radical anion which can be metabolized in two ways (Figure 7). First, the free-radical anion can be re-oxidized to the inactive prodrugs by molecular oxygen with the production of superoxide. Thereby, molecular oxygen reverts the reduction step and these nitroreductases are, therefore, called „oxygen-sensitive". This process of reduction and re-oxidation is repeated endlessly and it is called 'futile cycling'. The toxic compound produced during this `futile cycling' is superoxide (O^-_2), produced which can easily be eliminated by superoxide dismutase and catalase. The second way to metabolize the toxic free radical anion is another one-electron transfer step to the more toxic nitrosoderivate that leads to DNA damage (26, 37).

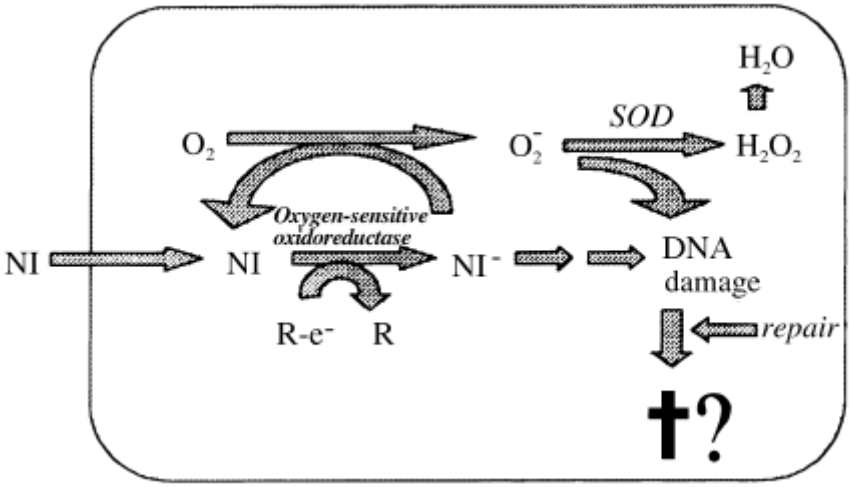

Figure 7. Metabolism of a nitroimidazole (NI) by an oxygen-sensitive nitroreductase

The acquisition of resistance is highly associated with mutational inactivation of the *rdxA* gene, which encodes an oxygen-insensitive NADPH nitroreductase. Recent evidence has suggested that inactivation of *frxA* (NADPH flavin oxidoreductase), *fdxA* (ferredoxin-like protein) and possibly other reductase-encoding genes may also contribute to the resistant phenotype.

However, it soon became apparent that the mechanism of metronidazole resistance in *H. pylori* was even more complex, as indications for the involvement of other nitroreductase-encoding genes associated. Mutational changes of the *frxA* and *fdxA* genes may contribute to metronidazole resistance. Recent evidence has suggested that inactivation of other nitroreductase-encoding genes including *frxA* (NADPH flavin oxidoreductase) and *fdxA* (ferredoxin-like protein) may contribute to the resistance phenotype (4, 38-48).

9. EFFLUX PUMP IN HELICOBACTER PYLORI (HEFA)

The efflux system is a mechanisms of intrinsic drug resistance involve decreased drug uptake or increased drug efflux . The resistance-nodulation-division (RND) family (Figure 8) of efflux systems is the one of five families of efflux system widespread in Gram-negative bacteria. Multidrug transporters belonging to the RND family interact with a membrane fusion protein (MFP) and an outer membrane protein to allow drug transport across both the inner and outer membrane of Gram-negative bacteria. The secondary structure of RND-type efflux proteins was proposed to consists of 12 TMS (49), and an outer membrane efflux protein (OEP) which is the TolC (the outer membrane efflux protein in *Escherichia coli*) or a TolC homolog, it is a multidrug efflux that can be pump out an extremely wide range of substrates, including practically all lipophilic and amphiphilic antibiotics, chemotherapeutic agents, metabolic inhibitors such as cerulenin, dyes, detergents (including SDS, Triton X-100, and bile salts), and solvents, especially can be pump metronidazole by proton pump inhibitor. The RND family efflux systems are dependent on the proton motive force. Such systems, including the AcrAB-TolC system in *Escherichia coli* , the AcrAB system in *Haemophilus influenzae* , the MexAB-OprM system in *P. aeruginosa*,(9-11, 50). Previous study has identified in *H. pylori* 11637 that presence of portions of three genes with homology to potential RND efflux systems and confirmed that *H. pylori* contained only these three putative RND efflux systems, *hefABC*, *hefDEF*, and *hefGHI*, and that the *hefGHI* system was expressed only *in vivo* while other two RND systems were expressed both in vivo and in vitro(9). Phylogenetic analysis of the *H. pylori* efflux homologs with other characterized RND

efflux systems revealed that the HefDEF and HefGHI systems were most similar to those systems involved in the efflux of divalent cations, while HefABC was most similar to those systems characterized as multiple-drug efflux pumps. However, it was apparent that, e.g., the *H. pylori* RND pump proteins are divergent (i.e., branch earlier on the phylogenetic tree) from other bacterial sequences. Of the three putative efflux systems identified in *H. pylori*, only one, HefABC, showed any homology to the RND efflux systems involved in multidrug resistance in other bacteria, and suggest that HefA efflux pump, TolC homolog may play a role in metronidazole resistance mechanism (10, 11).

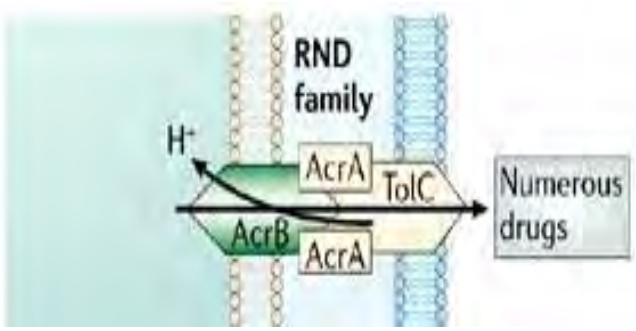

Figure 8. A diagrammatic representation of the structure and membrane location of RND family efflux pumps (49).

CHAPTER IV

MATERIALS AND METHODS

Methodology Scheme

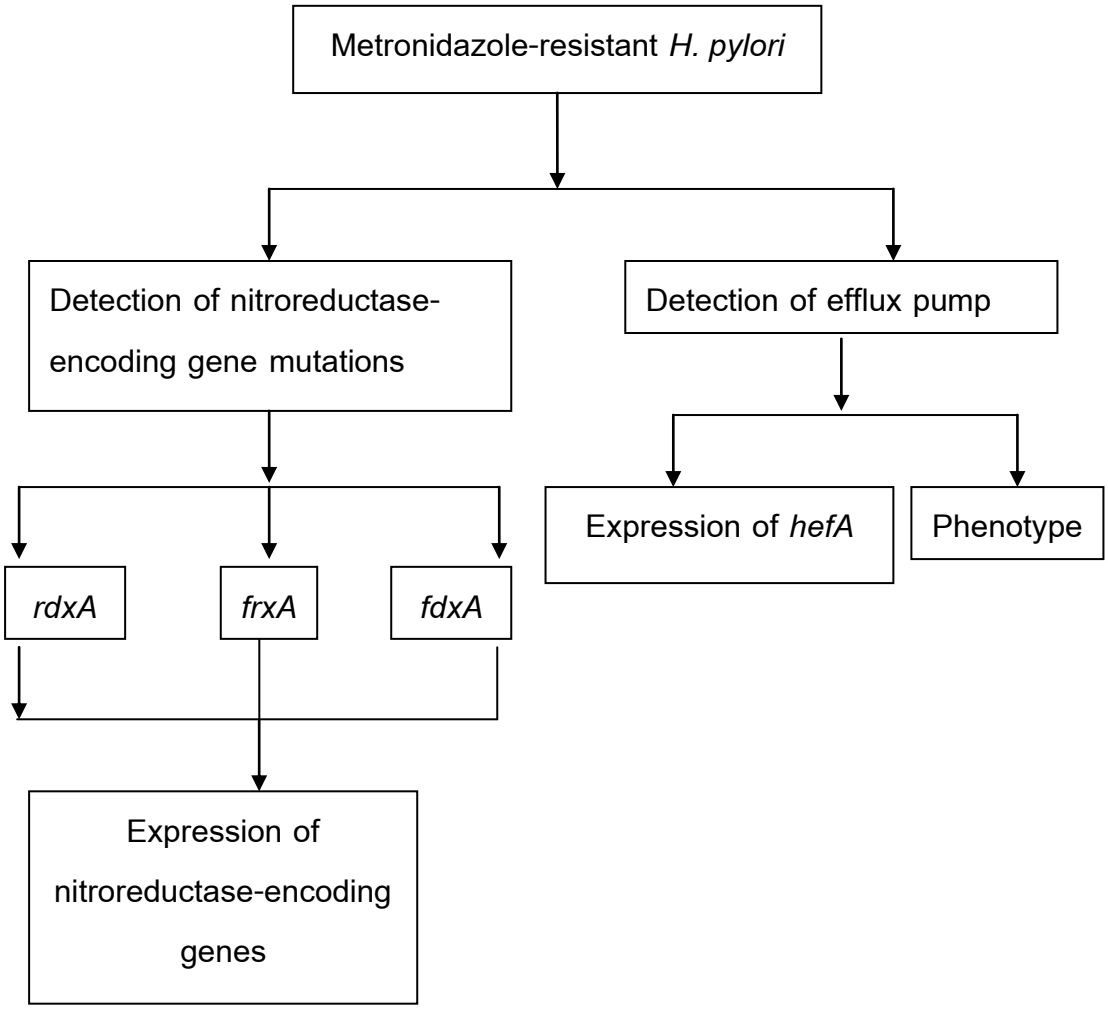

Figure 9. Methodology Scheme

PART I : CLINICAL ISOLATES

1. BACTERIAL STRAINS

Thirty metronidazole-resistant *Helicobacter pylori* and five metronidazole-susceptible isolates were obtained from a collection culture stored at -70 ^{o}C at the Department of Microbiology, Faculty of Medicine, King Chulalongkorn Memorial Hospital (Bangkok).

2. PRESERVATION OF *H. PYLORI* ISOLATES

All *H. pylor*i isolates were grown on Columbia blood agar with 7% sheep blood and 7% horse serum (GibcoBRL, U.S.A.) at 37 ^{o}C under microaerobic condition for 3 days. The colonies were transferred by loop into cryogenic vials containing brain heart infusion broth (Oxoid, England) containing 20% glycerol (V/V)and were kept at -70 ^{o}C until used.

PART II : DETECTION OF NITROREDUCTASE-ENCODING GENES MUTATIONS

Nitroreductase-encoding genes including *rdxA*, *frxA* and *fdxA* were amplified by PCR and then sequenced.

1. DNA extraction

The extraction of *H. pylori* was performed by QIAamp DNA Mini Kit (Qiagen, Germany). The DNA was purified according to manufacturor‟s directions. *H. pylori* colonies were scraped from the culture plate with an inoculation loop and suspended in 180 µl of buffer ATL (supplied in the QIAamp DNA Mini Kit). Twenty microlitres of Proteinase K were added, mixed by vortexing, and incubated at 56°C until bacterial cell were completely lysed. Lysis is usually complete in 1–3 h. The sample was then added with 200 µl of buffer AL, mixed by pulse-vortexing for 15 s, and incubated at 70 °C for 10 min and briefly centrifuged to remove drops from inside

the lid. Then, 200 µl ethanol (100%) was added to the sample, and mixed by pulse-vortexing for 15 s. The mixture including the precipitate were transferred to the QIAamp spin column in a 2 ml collection tube. and centrifuged at 8000 rpm for 1 min. the QIAamp spin column were placed in a clean 2 ml collection tube, and the filtrate was discarded. the QIAamp spin column were carefully opened and 500 µl of buffer AW1 were added and centrifuged at 8000 rpm for 1 min. QIAamp spin column were placed in a clean 2 ml collection tube, and the filtrate was discarded. Five hundred µl of buffer AW2 were add to QIAamp spin column, and centrifuge at 14,000 rpm for 3 min. The QIAamp spin column was placed in a clean 1.5 ml microcentrifuge tube and was added with 200 µl Buffer AE. The sample were incubated at room temperature for 1 min, and then centrifuged at 8000 rpm for 1 min. The extracted DNA was stored at -20 ^{o}C.

2. Primers for PCR and DNA sequencing

PCR are shown in Table 4 used for Amplified *rdxA*, *frxA* and *fdxA* genes

Table 4. Primers used for amplification of *rdxA*, *frxA* and *fdxA* genes

Gene	Primer	Primer sequence (5'- 3')	Product size (bp)	Reference
rdxA	*rdxA*-F	5‟-ATGGGTTGCTGATTGTGGTTTATGG-3‟	947 BP	(51)
	rdxA-R	5‟-AAAACACCCCTAAAAGAGCG-3‟		
frxA	*frxA*-F	5‟CGAATTGGATATGGCAGCCG-3‟	910 BP	(41)
	frxA-R	5‟-TATGTGCATATCCCCTGTAGG-3‟		
fdxA	*fdxA*-F	5‟-CGCTTGTTCAAGGCTCTGATG -3‟	826 BP	(43)
	fdxA-R	5‟-CGCTACAAACTCCAGCCGATT-3‟		
hefA	*hefA-F*	5‟ACGCCTCGAGTAAAAGCGCAAGGGAATTTG-3‟	1788 BP	this study
	hefA-R	5‟ACGCTCTAGATTCGCTAATTGGCCTAGCAT-3‟		

3. Amplification of *rdxA* gene by PCR

Primer RdxAF1 and RdxAR1 were used for amplification the entire *rdxA* gene in 50 µl PCR reaction mixture containing 1X *Taq* buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs (Fermentas, USA), 0.2 µM of each forward and reverse primer, and 1 U *Taq* polymerase (Fermentas, USA), and 1 µL of DNA template. The amplification conditions were, initial denaturation at 94 ºC for 1 minutes, 35 cycles of 94 ºC for 40 sec. , 57 ºC for 40 sec., and 72 ºC for 1 minute, and a final elongation at 72 ºC for 10 minutes.

4. Amplification of *frxA* gene by PCR

Primer *frxA*-F and *frxA*-R were used for amplification the entire *frxA* gene in 50 µl PCR reaction mixture containing 1X *Taq* buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs (Fermentas, USA), 0.2 µM of each forward and reverse primer, and 1 U *Taq* polymerase (Fermentas, USA), and 2 µL of DNA template. The amplification conditions were, initial denaturation at 94 ºC for 30 minutes, 35 cycles of 94 ºC for 30 sec. , 57 ºC for 30 sec., and 72 ºC for 1 minute, and a final elongation at 72 ºC for 10 minutes.

5. Amplification of *fdxA* gene by PCR

Primer *fdxA* -F and *fdxA* -R were used for amplification the entire *fdxA* gene in 50 µl PCR reaction mixture containing 1X *Taq* buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs (Fermentas, USA), 0.2 µM of each forward and reverse primer, and 1 U *Taq* polymerase (Fermentas, USA), and 2 µL of DNA template. The amplification conditions were, initial denaturation at 94ºC for 30 minutes, 35 cycles of 94ºC for 30 sec. , 57 ºC for 30 sec., and 72 ºC for 1 minute, and a final elongation at 72ºC for 10 minutes.

6. Analysis of amplified DNA

The PCR products were analyzed on 1.0% agarose (Pronalisa, Spain) in 0.5X TBE buffer containing 0.5 μg/ml of ethidium bromide (Sigma, USA). PCR products were mixed with 6X of loading dye buffer (20% ficoll, 0.05% bromphenol blue). The electrophoresis was carried out at 100 volts for 50 minutes. The amplified products were visualized and photographed under UV light transilluminator. A 100 bp DNA ladder (Fermentus, USA) was used as a DNA size marker.

7. Purification of PCR products

The PCR products of *rdxA, frxA* and *fdxA* genes were purifired by QIAquick PCR purification kit as described by the manufacturer (QIAGEN , Max-Volmer-StraBe4 , Hilden, Germany). Five volume of Buffer PBI were added into the 1 volume of PCR products and mixed by pulse-vortexing. QIAquick spin column was placed in a provided 2 ml collection tube. The sample were then transferred to the 2 ml QIAquick spin column and centrifuged 13,000 rpm for 1 min. The filtrated was discarded and 750 ml of PE buffer were added into the QIAquick spin column, centrifuged for 1 min. Flow-through was discarded. QIAquick spin column were placed back in the same tube, and centrifuged for 60 sec. The QIAquick spin column was placed in a clean 1.5 ml microcentrifuge tube.Thirty microliters of buffer EB were added into the the QIAquick spin column in a clean 1.5 ml to eluted the pured DNA. The concentration of DNA was measured by spectrophotometer (BIO RAD, Smart Spec tm 3000, U.S.A) and approximately adjusted to 50-100 ng/μl for preparation of sequencing reaction. The purified PCR products were stored at -20^{o}C.

8. Preparation of sequencing reaction

Automated sequencing was done at the Macrogen Inc. (Seoul, Korea). Sequencing reaction was done by the chain termination method. DNA samples were sequenced both directions forward and reverse primers using the two primer. Sequencing was conducted under BigDyeTM terminator cycling conditions. The

reacted products were purified by ethanol precipitation and running using automatic sequencer, Applied Biosystems DNA sequencer model 3730xI (Rochester NY, USA).

9. **Sequence analysis**

The nucleotide sequences and the deduced amino acid sequences were analyzed with the software available over the Internet at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST) and ExPASy (www.expasy.org/). Multiple sequence alignment of sequences were analyzed by Multalin (http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html) and BioEdit program.

PART III : DETECTION OF THE EXPRESSION OF NITROREDUCTASE-ENCODING GENES AND EFFLUX PUMP HEFA

1. RT-PCR analysis of mRNA levels.

1.1 RNA EXTRACTION

(i) HOMOGENIZATION Exponentially growing *H. pylori* colonies were suspended in BHI broth (10^7 cells) and centrifuged 12000 g and lysed cells in 1ml TRIZOL® Reagent (Invitrogen Cat. No. 15596-018) by repetitive pipetting. Use 1 ml of the reagent per 1×10^7 bacterial cells. Washing cells before addition of TRIZOL® Reagent should be avoided as this increases the possibility of mRNA degradation.

(ii) PHASE SEPARATION Incubate the homogenized samples for 5 minutes at 15 to 30°C to permit the complete dissociation of nucleoprotein complexes. Add 0.2 ml of chloroform per 1 ml of TRIZOL Reagent. Cap sample tubes securely. Shake tubes vigorously by hand for 15 seconds and incubate them at 15 to 30°C for 2 to 3 minutes. Centrifuge the samples at no more than 12,000 × *g* for 15 minutes at 2 to 8°C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The volume of the aqueous phase is about 60% of the volume of TRIZOL Reagent used for homogenization.

(iii) RNA PRECIPITATION Transfer the aqueous phase to a fresh tube, and save the organic phase if isolation of DNA or protein is desired. Precipitate the RNA from the aqueous phase by mixing with isopropyl alcohol. Use 0.5 ml of isopropyl alcohol per 1 ml of TRIZOL Reagent used for the initial homogenization. Incubate samples at 15 to 30°C for 10 minutes and centrifuge at no more than 12,000 × *g* for 10 minutes at 2 to 8°C. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube. (iv) RNA WASH Remove the supernatant. Wash the RNA pellet once with 75% ethanol, adding at least 1 ml of 75% ethanol per 1 ml of TRIZOL Reagent used for the initial homogenization. Mix the sample by vortexing and centrifuge at no more than 7,500 × *g* for 5 minutes at 2 to 8°C. (v) REDISSOLVING THE RNA At the end of the procedure, briefly dry the RNA pellet (air-dry for 5-10 minutes). It is important not to let the RNA pellet dry completely as this will greatly decrease its solubility. Partially dissolved RNA samples have an A260/280 ratio < 1.6. Dissolve RNA in RNase-free water or 0.5% SDS solution by passing the solution a few times through a pipette tip ~ 15-20 µl, and incubating for 10 minutes at 55 to 60°C finally storage the RNA at -80°C

1.2 RT-PCR

To convert the RNA to cDNA by Superscript III strand Synthesis System for RT-PCR Invitrogen (Cat.No. 18080-051)

First-Strand cDNA Synthesis

The following procedure is designed to convert 1 pg to 5 µg of total RNA or 1 pg to 500 ng of poly(A)+ RNA into first-strand cDNA: Mix and briefly centrifuge each component before use. Then combine the following in a 0.2- or 0.5-ml tube: (Component Amount up to 5 µg total RNA *n* µl , Primer* 1 µl, 50 ng/µl random hexamers, 10 mM dNTP mix 1 µl, DEPC-treated water to 10 µl) Then incubate at 65°C for 5 min, and place on ice for at least 1 min. After that prepare the following cDNA Synthesis Mix, adding each component in the indicated order: (Component 1 Rxn 10 Rxns,10X RT buffer 2 µl 20 µl, 25 mM MgCl2 4 µl 40 µl, 0.1 M DTT 2 µl 20 µl, RNaseOUT. (40 U/µl) 1 µl 10 µl, SuperScript. III RT (200 U/µl) 1 µl 10 µl). Then add 10 µl of cDNA Synthesis Mix to each RNA/primer mixture, mix gently, and

collect by brief centrifugation. Incubate as follows. Oligo(dT)20 or GSP primed: 50 min at 50°C Random hexamer primed: 10 min at 25°C, followed by 50 min at 50°C. Then terminate the reactions at 85°C for 5 min. Chill on ice. Then collect the reactions by brief centrifugation. Add 1 µl of RNase H to each tube and incubate for 20 min at 37°C. and finally cDNA synthesis reaction can be stored at -20°C or used for PCR immediately.

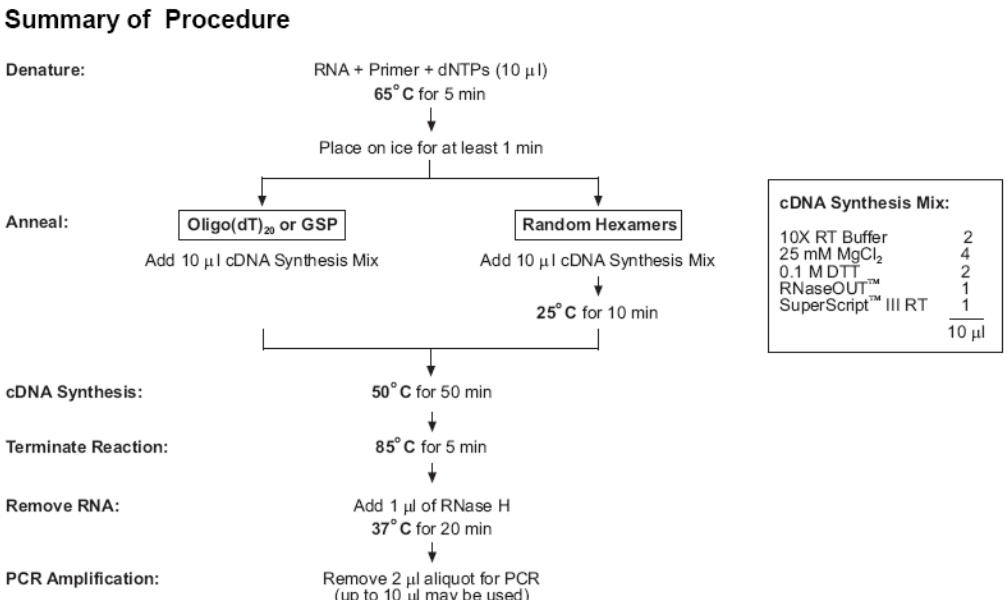

Figure 10. Summary procedure of RT-PCR

PART IV : DETECTION OF EFFLUX PUMP PHENOTYPE

1. Detection of efflux pump in metronidazole-resistant *H. pylori* by efflux inhibitor, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP)

To study inhibitory effects of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) against efflux, susceptibility testing was carried out using agar dilution method. MIC changes were observed in either the absence or the presence of CCCP (Sigma, USA) at concentrations of 100 μM, as described by (52). The Mueller Hinton agar containing the two-fold dilutions of metronidazole were inoculated with $5x10^4$ cfu/ml of each isolate. Plates were incubated at 37^oC under microaerophilic condition for 3 days. A phenotype for positive efflux was detectable after at least four fold of metronidazole MICs in the the presence of CCCP.

CHAPTER V

RESULTS

PART I : BACTERIAL STRAINS

A collection of thirty isolates of metronidazole-resistant *H. pylori* and five metronidazole-susceptible isolates were used in this study. They were obtained from gastric biopsy specimens and kept at the Department of Microbiology, King Chulalongkorn Memorial Hospital, Bangkok, Thailand between August 2003 and June 2004. The isolates were stored at -80^{o}C

PART II: AMPLIFICATION OF RDXA GENE BY POLYMERASE CHAIN REACION

Thirty five *H. pylori* isolates were screened for the presence of *rdxA* gene by PCR with primers RdxA-F and RdxA-R. Of the 35 *H. pylori* isolates, 5 were susceptible to metronidazole (MIC range = 0.016 – < 8 μg/ml) and 30 were resistant to metronidazole (MIC range = 32- ≥256μg/ml). PCR products of 947 bp were present in all isolates. Figure 11 shows PCR products of metronidazole-susceptible isolates (lane 1-3) and metronidazole– resistant isolates (lane 4-9).

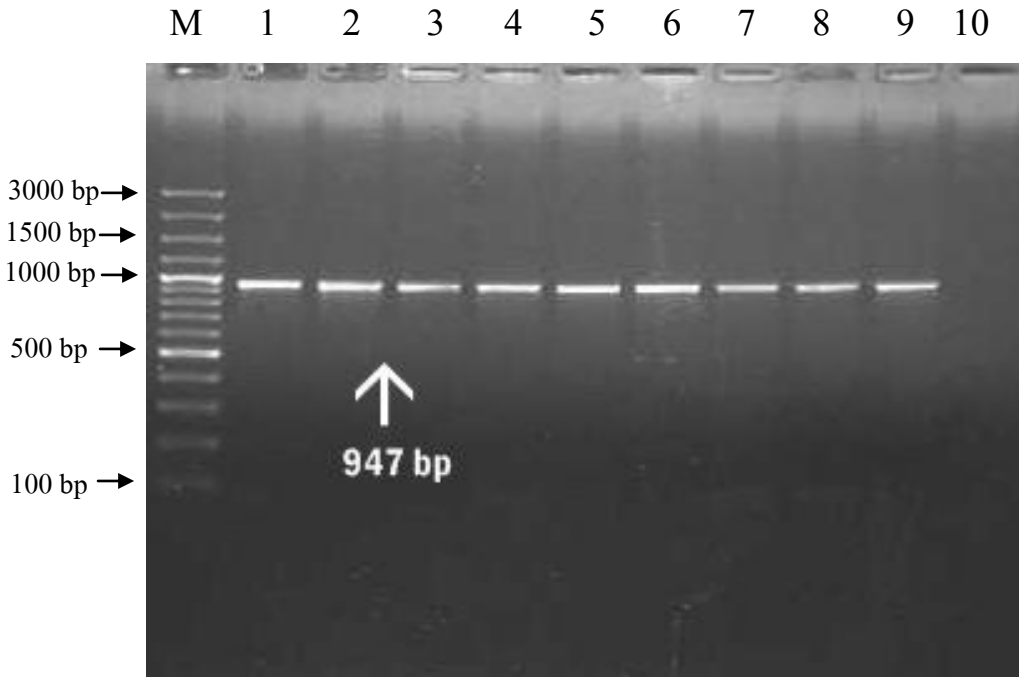

Figure 11. Electrophoresis of *rdxA* PCR products

Lane M : 100 bp plus DNA marker

Lane1-9 : metronidazole-susceptible (H30, H37, H57) and metronidazole-resistant *H. pylori* isolates (H31, H40, H47, H61, 464, H2)

Lane 10 : negative control (DDW).

PART III: AMPLIFICATION OF FDXA GENE BY POLYMERASE CHAIN REACION

Thirty five *H. pylori* isolates were screened for the presence of *rdxA* gene by PCR with primers FdxA-F and FdxA-R. Of the 35 *H. pylori* isolates, 5 isolales were susceptible to metronidazole (MICs range = 0.016 – < 8 μg/ml) and 30 isolates were resistant to metronidazole (MIC range =32- ≥256μg/ml). PCR products of 826 bp were present in all isolates. The PCR products of representative isolates are shown in Figure 12.

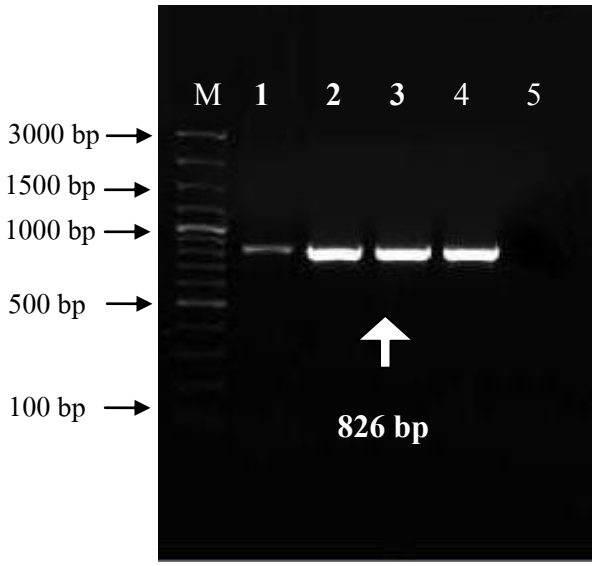

Figure 12. Electrophoresis of *fdxA* PCR products.

Lane M : 100 bp plus DNA marker

Lane 1-4 : metronidazole-resistant *H. pylori* isolates (H31, H40, H47 and H61)

Lane 5 : negative control (DDW).

PART IV: AMPLIFICATION OF FRXA GENE BY POLYMERASE CHAIN REACION

Thirty five *H. pylori* isolates were screened for the presence of *rdxA* gene by PCR with primers FrxA-F and FrxA-R. Of the 35 isolates, 5 were susceptible to metronidazole (MICs range = 0.016 – < 8 μg/ml) and 30 were resistant to metronidazole ((MIC range =32- ≥256μg/ml). PCR products of were present in all isolates, as shown in Figure 13.

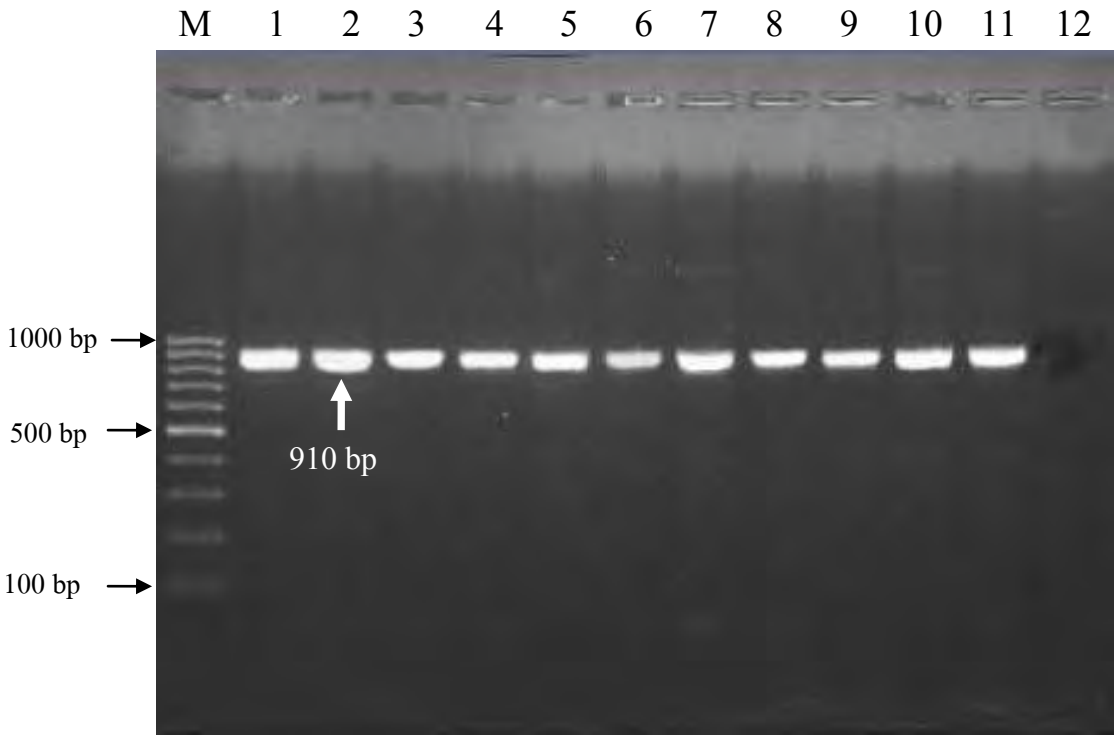

Figure 13. Electrophoresis of *frxA* gene PCR products by PCR.

Lane M : 100 bp DNA marker

Lane 1-11 : metronidazole-susceptible (H30, H37, H57,H74,H101) and metronidazole-resistant *H. pylori* isolates (H31, H40, H47, H61, 464, H2)

Lane 12 : negative control (DDW).

PART V: MUTATIONS IN RDXA

RdxA

To identify MTZ resistance-associated mutations in *rdxA* genes, PCR amplified *rdxA* containing fragments of the expected size, 947 bp, were sequenced from the 35 *H. pylori* isolates including 30 MTZ-resistant isolates (MIC ≥8 μg/ml) and 5 MTZ-sensitive isolates. Amino acid changes in RdxA are shown in (Table 5-14) and Appendix E.

The RdxA sequences of 30 MTZ-resistant isolates and 5 MTZ-susceptible isolates were compared with those of *H. pylori* 26695 (Genbank accession no. AE000573). There were 10-30 amino acid substitutions in RdxA among all isolates sequenced. Frameshift mutation was found in MTZ-resistant isolates, including H17, 876, C37, C66, TU970, 903, H3, G-3, G-8, TU851. In H17, there was mutation at amino acid position 8 that occurred by C insertion at nucleotide position 23, leading to stop codon at amino acid position 17. Premature stop condon was also found at amino acid position 29, 50, 65, 73, 76, 92, 111 and 166 in 9 MTZ-resistant isolates (876, C37, C66, TU970, 903, H3, G-3, G-8, TU851). Amino acid substitutions were found in both MTZ-susceptible and MTZ-resistant isolates. There were 31 amino acid substitutions, including Q6H, R16H/C, M21V, T31E/K, A37T, H53R, D59N, L62V, K64N, A67V, E75Q, A80T, S88P, R90K, P91S, H97Y/T, G98S, P106S/L, S108A, V111A, S128G, R131K, A143V, S152N, V172I, E175A/K/G, A183V, E194D, V204I, D205A, and A206T. Amino acid substitutions which caused premature stop codon were found in MTZ-resistant isolates, including H43, G-1, TU960, H47 and C94. In the isolate H43, there was stop codon at a amino acid position 50 that was occurred by nucleotide C substitution to T at nucleotide position 149. In G-1, TU960, H47 and C94, stop codon was found at amino acid position 65 which occurred by nucleotide C substitution to T at nucleotide position 194.

Of the 31 amino acid substitutions, 10 including Q6H, T31E, D59N, K64N, R90K, H97T, G98S, P106S, R131K, and V204I were shared by both metronidazole-resistant and –susceptible isolates (Table 5-14). Therefore, these amino-acid changes are probably not important for nitroreductase function. However, the variability of nucleotide substitutions and amino acid substitutions in the RdxA were not related to the metronidazole MICs, suggesting that these amino acid changes are not the only mechanism related to the functional activity of RdxA. Amino acid substitutions in RdxA in metronidazole-susceptible isolates suggested that there was genetic diversity among the *rdxA* gene of *H. pylori*.

Table 5. RdxA amino-acid changes in metronidazole-susceptible and -resistant *H. pylori* isolates

Strains	MIC	Amino acid changes position in RdxA																																									
Susceptible strains		6	8	16	17	21	29	31	37	50	53	56	59	62	64	65	67	68	73	75	76	80	88	90	91	92	97	98	106	108	111	118	128	131	143	162	166	172	175	183	194	204	205	206
26695*	0.06	Q	K	R	H	M	S	T	A	Q	H	M	D	L	K	Q	A	A	N	E	M	A	S	R	P	S	H	G	P	S	V		S	R	A	G	P	V	E	A	E	V	D	A
H30	0.38	.	.	.	.	.	.	E	.	.	.	.	N	.	N	.	.		.	.	.	.	.		.	.	T		S	.	.	.	.		.	.	.	.	.	.	.	I	A	.
H37	0.094	H	.	.	.	.	.	E	.	.	.	.	N	.	N	.	.		.	.	.	.	.	K	.	.	T		S	.	.	T	.	K	.	.	.	I	.	.	.	.	.	.
H57	0.094	H	.	.	.	.	.	E	.	.	.	.	N	.		.	.	V	.	.	.	.	.		.	.	.	.	.	.	.	.	.	K	.	.	.	I	.	.	.	.	.	.
H74	0.50	.	.	.	.	.	.	E	.	.	.	.	S	.		.	.		.	.	.	.	.	K	.	.	.	S	.	.	.	.	.		.	.	.	.	.	V	.	T	A	.
H101	0.25	.	.	.	.	.	.	E	.	.	.	.	N	.	N	.	.		.	.	.	.	.		.	.	T		.	.	.	.	.	K	.	.	.	.	Q		.	I	A	.
Resistant strains																																												
H111	>256	.	.	.	.	.	.		.	.	.	.	N		.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	K	.	.	.	.	.		.	.	.	T
H31	>256	.	.	.	.	.	.	E	.	.	.	.	N		.	.	.	.	.	.	.	.	P	.	.	.	.		.	.	.	.	.	K	.	.	.	.	.	V	.	.	.	.
H40	>256	.	.	.	.	.	.	E	.	.	R	.	N	V	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	.
H73	>256	.	.	H	.	.	.	E	.	.	.	.	N		.	.	.	.	.	.	.	.	.	K	.	.	Y		.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.
1173	64	.	.	H	.	.	.	E	.	.	.	.	N		.	.	.	.	.	.	.	.		K		.	Y		.	.	.	.	.	.	.	.	.	.	.	.	D	I	.	.
H61	192	.	.	H	.	.	.	.	.	.	R	.	N		.	.	.		.	.	.	.	P		.	.	.	S	.	.	.	.	.	K	V	.	.	.	.	.	.	.	.	T
c99	>256	.	.	H	.	.	.	.	.	.	R	.	N		.	.	.		.	.	.	.	P		.	.	.	S	.	.	.	.	.	K	V	.	.	.	.	.	.	.	.	T
464	32	.	.	C	.	.	.	E	.	.	R	.	N	V	.	.	.		.	.	.	.	P		.	.	.	S	.	A	.	.	.	K	.	.	.	.	.	.	.	.	.	T
H2	>256	.	.	C	.	V	.	E	.	.	R	.	N		.	.	V	.	.	.	.	.	P		S	.	.	S	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	T
H35	>256	.	.	H	.	.	.	E	T	.	R	.	N	V	.	.	.	.	.	.	.	.	.	.	.	.	.	S	L	.	.	.	.	K	.	.	.	.	.	.	.	I	A	.
H43	>256	.	.	.	.	.	.	E	.	_a																																		
g-1	>256	.	.	.	.	.	.	E	.	.	R	.	N	V	.	_a																												
TU960	>256	.	·	.	.	.	.	E	.	.	R	.	N	V	.	_a																												
H47	64	.	·	.	.	.	.	E	.	.		.	N	.	.	_a																												

*; GenBank accession no.AE000511 , **.** ; Same as for 26695, _[a]; amino acid substitution leading to stop codon, _[b]; Frameshift mutation leading to stop codon, _c ; stop codon

Table 5(continued). RdxA amino-acid changes in metronidazole-susceptible and -resistant *H. pylori*

Strains	MIC (μg/ml)	Amino acid changes position in RdxA																																										
Susceptible strains		6	8	16	17	21	29	31	37	50	53	56	59	62	64	65	67	68	73	75	76	80	88	90	91	92	97	98	106	108	111	118	128	131	143	162	166	172	175	183	194	204	205	206
26695*	0.06	Q	K	R	H	M	S	T	A	Q	H	M	D	L	K	Q	A	A	N	E	M	A	S	R	P	S	H	G	P	S	V		S	R	A	G	P	V	E	A	E	V	D	A
Resistant strains																																												
C94	>256	.		.	.	.	.	E	.	.	.	.	N	.	.	_a																												
H98	128	H		.	.	.	.	E	.	.	.	.	N	.	.	.	.	.	.	Q	.	T	.		.	.	T		S	.	.	.	G	K	.	.	.	.	.	.	.	.	.	.
C71	>256	.		.	.	.	.	E	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.		.	.	T		S	.	A	.	.		.	.	.	.	.	.	.	I	A	.
H12	>256	.		C	.	.	.		.	.	R	.	N	V	.	.	.	.	.	.	.	.	.		.	.	T		S	.	.	.	.	K	.	.	.	.	.	.	.	.	.	T
H80	>256	.		C	.	.	.		.	.	R	.	N	V	.	.	.	.	.	.	.	.	.		.	.	T		S	.	.	.	.	K	.	.	.	.	.	.	.	.	.	T
g-3	>256	.		.	.	.	.	E	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	K	.	.	.	S	.	.	.	.	.	K	.	W[b]	_c							
g-8	>256	.		.	.			E	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	K	.	.	.	S	.	.	.	.	.	K	.	W[b]	_c							
TU851	>256	.		.	.	.	.	E	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	K	.	.	.	S	.	.	.	.	.	K	.	W[b]	_c							
828	>256	.		.	.	.	.	E	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	K	.	.	T		.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	.
H3	>256	.		.	.	.	.	E	.	.	.	.	N	.	.		.	.	.	.	.	.	.		.	.	.		.	.	.	.	.	K	.	E[b]	_c							
H17	>256	.	T[b]		_c																																							
903	>256	.		.	.	.	.	E	.	.	.	T	N	.	.	T[b]															_c													
C37	>256	.		.	.	.	.	K	.	.	.		N	.	.	T[b]			_c																									
C66	>256	H		.	.	.	.	I	.	.	.		N	.	.	T[b]					_c																							
TU970	>256	.	.	.	.	.	.	E	.	.	.		N	.	.	T[b]			_c																									
876	>256	H	.	.	.	.	I[b]																	_c																				

*; GenBank accession no.AE000511 , . ; Same as for 26695, _[a]; amino acid substitution leading to stop codon, _[b]; Frameshift mutation leading to stop codon, _c ; stop codon

PART VI: MUTATIONS IN FDXA

Metronidazole resistance-associated mutations in the *fdxA* gene were determined all 35 isolates studied. PCR-amplified *fdxA* containing fragments of the expected size, 826 bp, were sequenced. Amino acid changes in FdxA are shown in Table 15 and Appendix. Isolate 828, a metronidazole-resistant isolate, contained a single missense mutation within the *fdxA* gene, Asn_{32} to His, when compared with amino acid sequences of *H. pylori* 26695. Six isolates including 1173, C94, G-3, 903, C37, and C66 had amino acid deletion at Asp_{47} that occurred by nucleotide TGA deletion at nucleotide position 138-140 (Table 15) and Appendix E.

Table 6. Amino acid changes in FdxA in metronidazole-susceptible and metronidazole-resistant *H. pylori* isolates

Strains	MICs (μg/ml)	Amino acid changes at position	
Susceptible strains		32	47
26695	0.06	N	D
H30	0.38	.	.
H37	0.094	.	.
H57	0.094	.	.
H74	0.50	.	.
H101	0.25	.	.
Resistant strains			
H111	>256	.	.
H31	>256	.	.
H40	>256	.	.
H73	>256	.	.
1173	64	.	-*
H61	192	.	.
C99	>256	.	.
464	32	.	.
H2	>256	.	.
H35	>256	.	.
H43	>256	.	.
G-1	>256	.	.
TU960	>256	.	.
H47	64	.	.
C94	>256	.	-*
H98	128	.	.
C71	>256	.	.
H12	>256	.	.
H80	>256	.	.
G-3	>256	.	-*
G-8	>256	.	.
TU851	>256	.	.
828	>256	H	.
H3	>256	.	.
H17	>256	.	.
903	>256	.	-*
C37	>256	.	-*
C66	>256	.	-*
TU970	>256	.	.
876	>256	.	.

*; GenBank accession no.AE000511 , **.**; Same as for 26695, -; deletions in amino acid

PART VII: MUTATIONS IN FRXA

As FrxA has been suggested to play a role in MTZ resistance, we analyzed the FrxA amino-acid sequences to identify any amino-acid changes. The FrxA amino acid sequences from *H. pylori* 26695 were used to compare with those of the 5 MTZ-susceptible and 30 MTZ-resistant isolates. Four of 5 MTZ-susceptible *H. pylori* encoded full-length FrxA, containing 1-4 amino acid substitutions. Truncated FrxA due to a frameshift mutation was found in 8 MTZ-resistant strains, including C37, H2, 903, C99, TU970, 828, H12 and H80 at amino acid position 4, 9, 18, 70, and 184, leading to premature stop codon at amino acid position 7, 29, 19/39, 73/88 and 216 respectively. Amino acid substitutions in 8 MTZ-resistant isolates (H73, 1173, H61, 464, H17, H40, C94 and TU851) which caused premature stop codon were observed at amino acid position 5 , 68, 73, 176, 84, and 101 respectively. Five of amino acid substitution positions was found in both metronidazole-susceptible and metronidazole–resistant isolates.

Table 7. FrxA amino-acid changes in metronidazole **-susceptible and -resistant *H. pylori* isolates**

Strains	**MIC (μg/ml)**	**Amino acid changes position in FrxA**																																						
Susceptible strains		4	5	7	9	11	15	16	18	19	26	29	39	40	41	43	44	68	70	71	72	73	84	85	88	101	111	117	124	131	154	162	176	184	193	204	206	208
26695*	0.06	E	Q	V	L	H	A	A	K	Y	S	D	L	A	P	S	I	W	A	L	F	G		A		H	N	I	N	E	A	P	E	A	C	K	R	K
H30	0.38	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	H	.	S	.	.	.	.	.	.	.	.	.
H37	0.094	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	H	.	S	.	.	.	.	.	S	.	.	.
H57	0.094	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H74	0.50	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	K	.	.	.	.	.
H101	0.25	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Resistant strains																																						
H111	>256	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H31	>256	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	K	.	S	E	.	.
H40	>256	.	.	I	.	.	.	T	.	.	.	.	.	.	.	.	E	.	.	.	S	S	.	.	.	.	D	M	S	G	.	.	a _					
H73	>256	.	a _																																				
1173	64	.	a _																																			
H61	192	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	_a																				
C99	>256	.	.	.	.	.	.	.	N	T	P	I	_c																									
464	32	.	.	.	.	.	.	T	.	.	.	.	.	.	.	A	E	.	G	Y	.	a _																
H2	>256	.	.	.	Y							_c																											
H35	>256	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.	V	S	S	.	.	.	.	.	.	S	.	.	.	K	.	S	.	.	.
H43	>256	.	.	.	.	.	.	T	.	.	.	.	.	V	.	A	E	.	.	.	S	S	.	.	.	.	D	M	S	.	V	.	.	.	S	.	.	N
g-1	>256	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Q	K	.	S	.	.	.
TU960	>256	.	.	.	.	.	.	T	.	.	.	.	.	V	.	A	E	.	.	.	S	S	.	.	.	.	D	M	S	.	V	.	.	.	S	.	.	N
H47	64	.	.	.	.	Y	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.

*; GenBank accession no.AE000511 , **. ;** same as for 26695, _[a]; amino acid substitution leading to stop codon, _[b]; Frameshift mutation leading to stop codon, _c ; stop codon

Table 7. FrxA amino-acid changes in metronidazole **-susceptible and -resistant *H. pylori* isolates**

Strains	**Amino acid changes position in FrxA**																																						
Susceptible strains	MIC	4	5	7	9	11	15	16	18	19	26	29	39	40	41	43	44	68	70	71	72	73	84	85	88	101	111	117	124	131	154	162	176	184	193	204	206	208
26695*	0.06	E	Q	V	L	H	A	A	K	Y	S	D	L	A	P	S	I	W	A	L	F	G	L	A	G	H	N	I	N	E	A	P	E	A	C	K	R	K
Resistant strains																																							
C94	>256	.	.	I	.	.	.	T	.	.	.	.	.	.	.	A	E	.	.	.	.	.	a_															
H98	128	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
C71	>256	.	.	.	.	.	T	.	.	.	.	.	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	,
H12	>256	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	M	.	.	.	.	.	G		_c		
H80	>256	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	D	M	.	.	.	.	.	G		_c		
g-3	>256	.	.	I	.	.	.	.	.	.	.	.	.	.	.	A	E	a_																				
g-8	>256	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	K		S	E	.	.
TU851	>256	.	.	I	.	.	.	T	.	.	.	.	.	.	.	A	E	.	.	.	S	S	.	.	.	a_												
828	>256	.	.	.	.	.	.		.	.	.	.	.	.	.	.	.	.	G	T		W			_c													
H3	>256	.	.	.	.	.	.	T	.	.	.	.	.	.	.	A	E	.	.	.	S	S	.	E	.	.	D	M	S	.	V	.	.	.	S	.	.	N
H17	>256	.	.	.	.	.	.	T	.	.	.	.	.	.	.	A	E	.	G	Y		_c																
903	>256	.	.	I	.	.	.	.	I	_c																												
C37	>256	D	K	c																																		
C66	>256	.	.	.	.	.	.	T	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
TU970	>256	.	.	.	.	.	.		N	T	P	I	_c																									
876	>256	.	.	.	.	.	.	.	.	..	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	K		S		C	

*; GenBank accession no.AE000511 , **. ;** Same as for 26695, _[a]; amino acid substitution leading to stop codon, _[b]; Frameshift mutation leading to stop codon, _c ; stop codon

Table 8. Alterations in RdxA, FdxA and FrxA in 35 *H. pylori* isolates

Isolates	MIC(μg/ml)	RdxA		FdxA		FrxA	
		Changes in nucleotide sequence	Changes in amino acid sequence	Changes in nucleotide sequence	Changes in amino acid sequence	Framshift mutation	Changes in amino acid sequence
H30	0.38	Missense mutation	T31E,D59N,K64N,H97T, P106S,V204I,D205A	-	-	-	A16T,I44V, N111H,N124S
H37	0.094	Missense mutation	Q6H,T31E,D59N,K64N,R90K, H97T,P106S,A118T,R131K,V172I	-	-	-	N111H,N124S,C193S
H57	0.094	Missense mutation	Q6H,T31E,D59N, A68V,R131K,V172I	-	-	-	V7I
H74	0.50	Missense mutation	T31E,D59S,R90K,G98S, A183V,V204T,D205A	-	-	-	A16T,E176K
H101	0.25	Missense mutation	T31E,D59N,K64N,H97T,R131K, E175Q,V204I,D205A	-	-	-	-
H111	>256	Missense mutation	D59N,G98S,R131K,A206T	-	-	-	A70V,C193S
H31	>256	Missense mutation	T31E,D59N,S68P, R131K,S152N,A183V	-	-	-	A16T,E176K,C193S,K204E
H40	>256	Missense mutation	T31E,D59N,S88P, R131K,S132N,A183V	-	-	-	V7I,A16T,I44E,F72S,G73S,N111D I117M,N124S,E131G, E176STOP
H73	>256	Missense mutation	R16H,T31E,D59N, H97Y,V204I	-	-	-	Q5STOP
1173	64	Missense mutation	R16H,T31E,D59N, R90K,H97Y,E194D,V204I	$TGA_{138\text{-}140}$deletion	Asp_{47} deletion	-	Q5STOP
H61	192	Missense mutation	R16H,H53R,D59N,S88P, G98S,R131K,A143V,A206T	-		-	V7I,W68STOP
C99	>256	Missense mutation	R16H,H53R,D59N,S88P, G98S,R131K,A143V,A206T	-	-	Frameshift,AA deletion at 54	K18N→L39STOP
464	32	Missense mutation	R16C,T31E,H53R,D59N,L62V, S88P,G98S,S108A,R131K,A206T	-	-	Frameshift,G_{211} insertion	A16T,S43A,I44E, A70G→G73STOP
H2	>256	Missense mutation	R16C,M21V,T31E,H53R,D59N, A67V,S88P,P91S,G98S,R131K,A206T	-	-	FrameshiftT_{26} deletion	L9Y→D29STOP

Table 8.(continued) Alterations in RdxA, FdxA and FxA in 35 *H. pylori* isolates

Isolates	MIC(μg/ml)	RdxA		FdxA		FrxA	
		Framshift mutation	Changes in amino acid sequence	Changes in nucleotide sequence	Changes in amino acid sequence	Framshift mutation	Changes in amino acid sequence
H35	>256	Missense mutation	R16H,T31E,A37T,H53R,D59N, G98S,P106L,R131K,V204I,D205A	-	-	Missense mutation	V7I,I44V,L71V,F72S,G73S, N124S,E170K,C193S
H43	>256	Missense mutation	T31E, Q_{50}STOP	-	-	Missense mutation	A16T,A40V,S43A,I44E,F72S,G73S,N111D I117M,N124S,A154V,C193S,K208N
G-1	>256	Missense mutation	T31E,H53R,D59N,L62V, Q65STOP	-	-	Missense mutation	A16T,P162Q,E176K,N193S
TU960	>256	Missense mutation	T31E,H53R,D59NL62V, Q65STOP	-	-	Missense mutation	A16T,A40V,S43A,I44E,F72S,G73S,N111D, I117M,N124S,A154V,C193S,K208N
H47	64	Missense mutation	T31E,D59N, Q65STOP	-	-	Missense mutation	H11Y,A16T,C193S
C94	>256	Missense mutation	T31E,D59N, Q65STOP	$TGA_{138\text{-}140}$deletion	Asp_{47} deletion	Frameshift TG, insertion 180	V7I,A16T,S43A, I44E,Q65STOP
H98	128	Missense mutation	Q6H,T31E,D29N,E75Q,A80T, H97T,P106S,S128G,R131K	-	-	-	A15T,P41L
C71	>256	Missense mutation	T31E,D59N,H97T,P106S, V111A,V204K,D205A	-	-	-	A15T,P41L
H12	>256	Missense mutation	R16C,H53R,D59N,L62V, H97T,P106S,R131K,A206T	-	-	Frameshift,G_{552} insertion	V7I,N111D,I117M A184G→K204STOP
H80	>256	Missense mutation	R16C,H53R,D59N,L62V,H97T, P106S,R131K,A206T	-	-	Frameshift,G_{552} insertion	V7I,A70V,N111D,I117M, A184G→K204STOP
g-3	>256	Frameshift,T_{489} insertion	T31E,D59N,R90K,G98S,R131K, G162W→P166STOP	$TGA_{138\text{-}140}$deletion	Asp_{47} deletion	-	V7I,S43A,I44E, W68STOP
g-8	>256	Frameshift,T_{489} insertion	T31E,D59N,R90K,G98S,R131K, G162W→P166STOP	$TGA_{138\text{-}140}$deletion	Asp_{47} deletion	-	A16T,E176K,G193S,K204E
TU851	>256	Frameshift,T_{489} insertion	T31E,D59N,R90K,G98S,R131K, G162W→P166STOP	-	-	Frameshift,A_{296} deletion	V7I,A16T,S43A,I44E, F72S,G73S,H101STOP

Table 8.(continued) Alterations in RdxA, FdxA and FxA in 35 *H. pylori* isolates

Isolates	MIC(μg/ml)	RdxA		FdxA		FrxA	
		Framshift mutation	**Changes in amico acid sequence**	**Changes in nucleotide sequence**	**Change in amico sacid sequence**	**Framshift mutation**	**Changes in amico acid sequence**
828	>256	Missense mutation	T31E,D59N,R90K, H97T,R131K	-	Asn_{32}→His	Frameshift,G_{211} insertion	A70G→G88STOP
H3	>256	Frameshift ,AG_{460} insertion	T31E,D59N,,R131K S152R→P166STOP	-	-	-	A16T,S43A,I44E,F73S,G73S,A85E,N111D, I117M,N124S,A154V,C193S,K208N
H17	>256	Frameshift,C_{23}insertion	K8T→H17STOP	-	-	Frameshift,G_{211} insertion	A16T,S43A,I44E, A70G→G73STOP
903	>256	Frameshift,T_{252}insertion	T31E,M56T,D59N, M84Y→V111STOP	$TGA_{138\text{-}140}$deletion	Asp_{47} deletion	Frameshift,AA deletion at 54	V7I K18I→Y19STOP
C37	>256	Missense mutation Frameshift,A_{195} insertion	T31K,D59N, Q65T→N73STOP	$TGA_{138\text{-}140}$deletion	Asp_{47} deletion	Frameshift,A_{12}deletion	E4D→V7STOP
C66	>256	Missense mutation Frameshift,A_{195} insertion	Q6H,T31K,D59N, Q65T→N73STOP	$TGA_{138\text{-}140}$deletion	Asp_{47} deletion	Frameshift,T_{78}deletion	A16T S26L→R58STOP
TU970	>256	Missense mutation Frameshift,A_{195} insertion	T31E,D59N Q65T→N73STOP	-	-	Frameshift,AA deletion at 54	K18N→L39STOP
876	>256	Missense mutation Frameshift,T_{85} deletion	Q6H S29I→S92STOP	-	-	-	A16T,E176K

Table 9. Conclusion Alterations in RdxA, FdxA and FxA in metronidazole-susceptible and-resistant *H. pylori* isolates

Strains	Amino acid changes position		
	RdxA	**FdxA**	**FrxA**
Susceptible- and resistant isolates	**Q6H** **V172I** **V204D** **T31E** D205A R90E **D59N** **R131K** H97T G98S **P106S** A183V	-	V7I A16T I44V **N111H** N124S E176K C193S
Resistant isolates	**R16H/C** M21V A37T **H53R** **M56T** **L62V** **A67V** **S88P** P91S S108A **V111A** A143V S152N E194D A206T	N32H Deletion at: D_{47}	A15T S26L A70V **F72S** G72S I117M
	Stop at: H17* **Q50** Q65 **N73*** **M76*** **S92*** **V111*** **P166**		Stop at: Q5 V7* Y19* D29* **L39*** R58* **W68** G73* L84* G88* H101 E176
Susceptible isolates	**A68D** **A118T** **E175Q**	-	-

_* : Frameshift mutation leading to stop codon , Blue label :amino acid change at position which was reported by other studies.

Table 10. The mutations in nitroreductase genes

Mutations in amino acid of	% of 30 MTZ-resistant isolates	% of 5 MTZ-susceptible isolates
RdxA	**100%**	**100%**
FdxA	**23.33%**	**0%**
FrxA	**100%**	**80%**
RdxA+FdxA	**23.33%**	**0%**
RdxA+FrxA	**100%**	**80%**
FrxA+FdxA	**23.33%**	**0%**
RdxA+FdxA+FrxA	**23.33%**	**0%**

PART VIII : ALTERATIONS IN THE UPSTREAM REGIONS OF RDXA FDXA AND FRXA GENES

The upstream regions from position -35 to -1, including the Shine Dalgarno (SD) sequence (AAGGAA) of the *rdxA* gene, had no alteration in 3 of the 5 MTZ-susceptible isolates, and 6 of the 30 MTZ-resistant isolates, when compared with susceptible strain 26695. One nucleotide, G at position -30 upstream from the start codon (ATG), was deleted in 8 metronidazole-resistant isolates. Nucleotide substitution from G to A was found in 17 of 30 metronidazole-resistant isolates and 1 metronidazole-susceptible isolates. Nucleotide substitution from A to G at nucleotide position -15 was only found in one of metronidazole-resistant isolate, TU960. A metronidazole-susceptible isolate, H30 had nucleotide substitution from A to G at nucleotide position -28 (Table 24).

For the upstream regions of the *fdxA* gene, a deletion of nucleotide T occurred at position -15 upstream from the gene and was found in 3 of the 5 metronidazole-susceptible isolates including H37, H57, H101 and 12 out of the 30 metronidazole-resistant isolates. Nucleotide substitution from T to G at position -5 was found in 3 metronidazole -resistant isolates including H40, 1173 and 903.

For the upstream regions of the *frxA* gene, a deletion of nucleotide T occurred at position -25 and -30 in both metronidazole-susceptible and metronidazole–resistant isolates. Two metronidazole-resistant isolates, G-1 and H47 was found 5‟-TTA-3‟ deletion at at nucleotide position (-3) to (-5). Nucleotide substitution from A to T at position -8 was found in 10 isolates (C99, H35, G-1, TU960, H47, C94, H17, C37, C66 and TU970) of 18 metronidazole-resistant isolates.

Table 11. Sequence variations in the upstream regions of *rdxA, fdxA and frxA* genes

***H. pylori* strains**	**MIC (mg/mL)**	**Gene upstream region sequences (from positions -35 to – 1)[a]**		
		***rdx*A gene**	***fdx*A gene**	***frx*A gene**
Susceptible strains				
26695	0.016	5‟-GCTACGAAAAATTCTAAAA AAATAAAGGAAAATCA-3‟	5‟-AAAGTCGTATTCAAACTTT TTAAAAGGAGTTAGTC-3‟	5‟-CGTTTATCATTATTTAGAAA AAGGAGAACATTAAA-3‟
H30	0.38	A-28G, G-30A	-	-
H37	0.094	-	T deletion at position -15	T deletion at position -25 T-5C
H57	0.094	-	T deletion at position -15	T deletion at position -30
H74	0.50	-	-	-
H101	0.25	G deletion at position -30	T deletion at position -15	-
Resistant strains				
H111	>256	-	T deletion at position -15	ND
H31	>256	G-30A	T deletion at position -15	ND
H40	>256	G-30A	T-5G	ND
H73	>256	G-30A	T deletion at position -15	-
1173	64	G-30A	T-5G	-
H61	192	G-30A	T deletion at position -15	T deletion at position -25
C99	>256	G-30A	T deletion at position -15	A-8T
464	32	-	-	ND
H2	>256	G-30A C-31T	-	T deletion at position -30
H35	>256	-	T deletion at position -15	A-8T
H43	>256	G-30A	-	ND
G-1	>256	-	-	5‟-TTA-3‟ deletion at position (-3) - (-5) A-8T
TU960	>256	G-30A, A-15G	-	A-8T

Table 11.(continued) Sequence variations in the upstream regions of *rdxA, fdxA and frxA* genes

H. pylori strains	MIC (mg/mL)	Gene upstream region sequences (from positions -35 to – 1)[a]		
		***rdx*A gene**	***fdx*A gene**	***frxA* gene**
Resistant strains				
H47	64	G deletion at position -30	T deletion at position -15	5‘‘-TTA-3‘‘ deletion at position (-3) – (-5) A-8T
C94	>256	G deletion at position -30	-	A-8T
H98	128	G-30A	-	T deletion at position -30
C71	>256	G-30A	-	ND
H12	>256	G-30A	T deletion at position -15	ND
H80	>256	G-30A	T deletion at position -15	ND
G-3	>256	G deletion at position -30	-	ND
G-8	>256	G deletion at position -30	-	ND
TU851	>256	G deletion at position -30		ND
828	>256	G deletion at position -30	A deletion at position -15	A-16G
H3	>256	G deletion at position -30	T-14C	A-8T T deletion at position -21
H17	>256	-	-	A-8T
903	>256	-	T-5G	ND
C37	>256	G-30A	-	A-8T
C66	>256	G-30A	T deletion at position -15	A-8T
TU970	>256	G-30A	-	A-8T
876	>256	G-30A	T deletion at position -15	ND

[a] Nucleotide A from the first codon (ATG) for *rdxA, fdxA* and *frxA* was counted as ‘‘-1’’, [b] ‘‘_’’; the same as that of strain 26695

Table 12. Summary of sequence variations in the upstream regions of *rdxA, fdxA and frxA* genes in metronidazole-susceptible and -resistant *H. pylori* isolates

Strains	Nucleotide changes position		
	rdxA	***fdxA***	***frxA***
Susceptible- and resistant strains	A-28G **G-30A**		T-5C
	Deletion at: G-30	Deletion at: T-15	Deletion at: T-25 T-30
Resistant strains	C-31T A-15G	T-5G T-14G	A-8T A-16G
	-	Deletion at: A-15	**Deletion at: 5'-TTA-3' (-3) T(-21)**
Susceptible strains	-	-	-

_* : Frameshift mutation leading to Stop codon , Blue label :amino acid change at position which was reported by other studies.

PART IX : DETECTION OF THE EXPRESSION OF NITROREDUCTASE-ENCODING GENES

1. RT-PCR analysis of mRNA levels.

The optimization of quantitative RT-PCR. The RT-PCR product (5 µl each) were loaded on 1.0% agarose gel containing 0.5 mg:ml ethidium bromide. Positive control with *H. pylori* H12 genomic DNA template was performed separately under the same condition. Negative control (RNA as template for frxA amplification) was performed under the same conditions as RT-PCR. RT-PCR products of *frxA* and *gyrB*(housekeeping gene used to compared the optical intensity with RT-PCR products of *frxA* gene) that detect by PCR with primers FrxA-F : FrxA-R and GyrB-F: GyrB-R. RT- PCR products of 910 bp and 267 bp were present respectively, as shown in Figure 14.

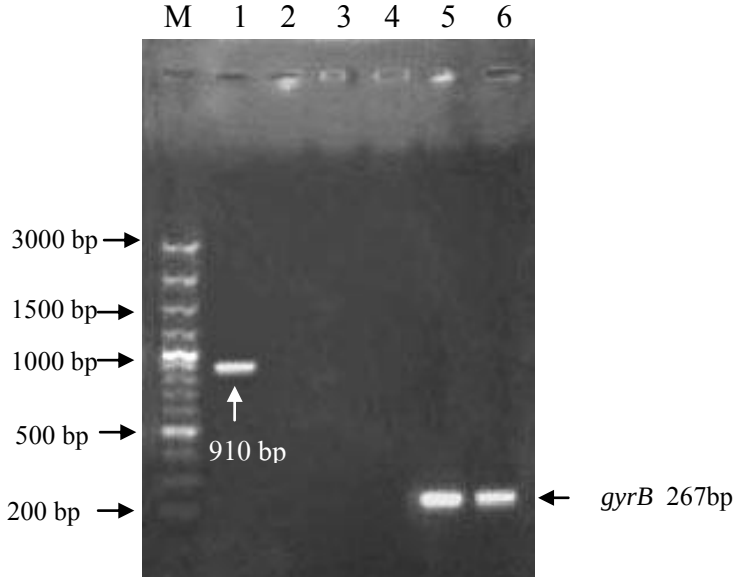

Figure 14. Electrophoresis of RT-PCR products of the *frxA* and *gyrB*

Lane M : 100 bp plus DNA marker

Lane 1 : PCR products of *frxA* gene ; Positive control with *H. pylori* H12 genomic DNA template

Lane 2 : RT-PCR products of *frxA* from H12

Lane 3 : negative control (RNA as template for *frxA* amplification)

Lane 4 : negative control (RNA as template for *gyrB* amplification)

Lane 5 : PCR products of *gyrB* gene ; Positive control with *H. pylori* H12 genomic DNA template

Lane 6 : RT-PCR products of *gyrB* from H12

PART X: DETERMINATION OF EFFLUX PUMP

The efflux pump mechanism was determined by agar dilution with or without the proton pump inhibitor, CCCP. Twelve metronidazole-resistant isolates with different MIC level were studiied. The decrease of metronidazole MIC of equal or greater than 4-fold is considered for the presence of efflux pump. There was no change in metronidazole MIC in the presence of CCCP in all tested isolates. The results showed that efflux pump was not present in any tested isolates. The results are summarized in Table 26.

Table 13. The effect of CCCP on 10 metronidazole-resistant *H. pylori.*

H. pylori strain	**No. of fold decreased**	**MIC range (ug/ml)**	
		no CCCP	CCCP
H74	0	0.5	0.5
H111	0	512	512
H31	0	512	512
H40	0	256	256
1173	0	64	64
C99	0	256	256
464	0	32	32
H2	0	512	512
H35	0	256	256
H43	0	512	512
G-1	0	512	512

The results showed that efflux pump metronidazole-resistant *H. pylori* isolates

CHAPTER VI

DISCUSSION

Helicobacter pylori is a gram-negative, microaerophilic, spiral bacterium that was first isolated from a stomach biopsy by Warren and Marshall in 1982. *H. pylori* is an important human pathogen that has now been accepted as the causative agent of chronic active gastritis, peptic ulcer diseases. Infection with *H. pylori* is also considered a risk factor for the development of gastric cancer including gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma. Triple therapy is highly effective for the treatment of *H. pylori* infection. However, bacterial resistance to one of the most effective antibiotics, metronidazole, is increasing reported.

Metronidazole is an antibiotic frequently included in treatment regimens for eradication of *H. pylori*. The efficacy of a metronidazole-containing regimen for the treatment of *H. pylori* infection is decreased by metronidazole resistance. The activity of metronidazole in *H. pylori* is dependent on the reduction of its nitro moiety to highly reactive compounds that cause DNA strand breakage. Metronidazole is metabolized by *H. pylori* by several nitroreductases of which an oxygen-insensitive NADPH nitroreductase, encoded by the *rdxA* gene was reported to be the most important. Goodwin *et al*. reported that mutations in *rdxA* gene were associated with metronidazole resistance (51). Inactivation of other nitroreductase-encoding genes including *frxA* (NADPH flavin oxidoreductase) and *fdxA* (ferredoxin-like protein) were observed to be involved in the resistance mechanism (38, 47)

This study examined the metronidazole resistance mechanism which focused on the contribution of the *rdxA, fdxA* and *frxA* genes. When compared with genes from metronidazole-sensitive isolates, *rdxA*, *fdxA* and *frxA* genes of metronidazole-resistant isolates were altered in the following way: nonsense mutations by directly introducing stop codons, and missense mutations by frameshift or nucleotide substitution. Results from this study suggest that the patterns of mutation in these genes have variation, and the changes in gene sequences are random.

Some of the mutations in the *rdxA* gene from metronidazole-resistant isolates in this study have been described by others. The Gln_6 to His mutation found in the *rdxA* gene of the metronidazole-resistant isolates was also present in resistant isolates

in the study by Solcà *et al.*(53). Arg_{16} to His was reported by Solcà *et al*., Chisholm SA *et al*., Kwon DH et al., Matteo MJ *et al*. and Yang YJ *et al.* (38, 46, 53-55). Frameshift mutation, leading to premature stop codon at N73 was also present in the studies by Marais *et al*. and Matteo M. J. *et al*. (41, 55). Amino acid substitution at Ser_{88} to Pro was reported by Kwon DH *et al.* and Solca NM *et al.*(38, 53). Amino acid substitution at Val_{172} to Ile and Val_{204} to Ile was found both in metronidazole-susceptible and –resistant isolates, similar to the study of Kwon DH *et al.*(38). In addition, we showed that a truncated RdxA protein could also result not only from a frameshift mutation but also from nonsense mutation by nucleotide substitution leading to stop codon. Of the 30 metronidazole-resistant isolates, truncated in RdxA was found in 11 isolates. In this study, we identified a variety of genetic alterations in *rdxA* associated with metronidazole resistance, most of which have not been described before.

One of the metronidazole-resistant strains, 828, contained single missense mutations in the *fdxA* gene at position 32 (Asn_{32} to His), and six isolates (1173, C94,G-3,903,C37,C66) had amino acid deletion at position 47 from nucleotide TGA deletion at position 138-140. All of metronidazole-susceptible isolates had no changes in amino acid sequences of FdxA (Table 15). The results suggest that the inactivation of FdxA protein may not be essential for metronidazole resistance in *H. pylori*.

In this study, we analyzed the FrxA amino-acid sequences to identify any amino-acid changes as it has been suggested to play a role in metronidazole resistance (41, 45). The study of Marais A. *et al*., suggested that metronidazole resistance phenotype may arise in *H. pylori* without mutations in *rdxA* or *frxA,* or with mutations only in *frxA.*(41) The FrxA amino acid sequences from 26695 was used to compare the FrxA sequences of the 35 clinical isolates; 5 strains of metronidazole-susceptible isolates and 30 metronidazole-resistant isolates. Among 15 metronidazole-resistant *H. pylori* isolates, 7 (C37, H2, C99, TU970, 828, H12, H80) had a frameshift mutation, leading to premature stop codon. Similar to our study, mutation at L39 leading to stop codon at C99 has been reported by Han F. *et al* (42). Four of the five metronidazole-susceptible isolates encoded full-length FrxA containing 1-4 amino-acids. However, Matteo MJ *et al.* showed that early truncation of FrxA was observed in both metronidazole-susceptible and metronidazole-resistant *H. pylori* isolates, suggesting that *frxA* may not play a major role in metronidozole resistance in *H. pylori* (55)

In addition, alterations in the upstream regions of *rdxA*, *fdxA* and *frxA* was investigated from position -35 to -1, which may contain the binding site of RNA polymerase or SD ribosome binding site that may influence the initiation of gene transcription. In upstream regions of *rdxA* gene, there were no alterations in 3 (H37, H57 and H74) of 5 metronidazole-susceptible isolates, of the 30 metronidazole-resistant isolates, 5 had no alterations in upstream regions. Nucleotide G at position -30 was deleted in 7 metronidazole-resistant isolates and nucleotide substitution from G to A was found in 17 metronidazole- resistant isolates. Han F. *et al.* was also found nucleotide deletion, but in different position from this study. From this results, suggesting that alterations in the upstream region of *rdxA* gene may be contributed to metronidazole resistance in *H. pylori*. For the upstream region of the *fdxA* gene, a deletion in nucleotide T occurred at position -15 upstream from the gene was found in 3 metronidazole-susceptible isolates and 12 metronidazole- resistant isolates and there was found nucleotide substitution from T to G at position -5 was present in 3 metronidazole-resistant isolates. For the *frxA* gene, a variation in nucleotide A to T occurred at position -8 upstream from the gene. This mutation was found in 10 of the 18 metronidazole-resistant isolates. Moreover, in the upstream regions of *frxA* genes from 2 metronidazole-resistant isolates (G-1 and H47), there were deletions of 5‟-TTA-3‟ between position -3 and -5. This multiple nucleotide polymorphisms were also report, by Han F. *et al.*(42). Nucleotide T deletion at position -25 and -30 in upstream regions of *frxA* were found in metronidazole-susceptible isolates and these changes were also found in 3 strains of metronidazole-resistant isolates suggesting that this mutation may not be contributed to the development of metronidazole resistance in *H. pylori*. Although the significance of these changes is now not fully understood.

Detection of the expression of nitroreductase-encoding genes were determined by RT-PCR. RT-PCR could not detect mRNA expression of nitroreductase genes and HefA in both susceptible and resistant isolates. A sensitive method, Real-time RT-PCR, is considered to be used for further investigation. The study by Kwon DH. *et al.* suggested that metronidazole resistance may also be acquired by decreasing the transcription of the genes involved in metronidazole reductive activation, in addition to the mutation in some individual genes such as *rdxA*(48).

Several previous evidence suggested that metronidazole resistance may not only be contributed by inactivation of nitroreductase encoding genes mechanisms.

Possible mechanism of intrinsic drug resistance involves decreased drug uptake or increased drug efflux. The resistance-nodulation-division (RND) family of efflux systems is one of the five families of efflux system that is widespread in gram-negative bacteria(9). In 2005 van Amsterdam K *et al*. suggested that the HefA (TolC homolog) efflux pump confered metronidazole resistance in *H. pylori* (10). In this study, CCCP could not reduce MIC in all of metronidaozole- resistant isolates. In contrast with the study by Liu. Z. Q.*et al*., the expression of *hefA* was higher in the induced multidrug resistant strains than in their parent strains(11). Our results showed that the inactivation of the HefA efflux pump by CCCP were not present in 10 tested metronidazole-resistant isolates

In this study, we characterized mechanisms of metronidazole resistance in *H. pylori* and showed that the mutation in RdxA, FdxA and FrxA were detected in metronidazole-resistant *H. pylori* isolates. Metronidazole-susceptible isolates had a few mutations in these genes which were shown by the study of Kwon *et al*. that, they may be involved in metronidazole resistance(38). We found various mutation in RdxA in metronidazole-resistant isolates and some mutation were also found in metronidazole-susceptible isolates which are in agreement with the study by Chisholm SA *et al*., suggesting that mutations in *rdxA* may not always be essential for metronidazole resistance(54). The number of mutations in nitroreductase-encoding genes may not associated with MIC level of metronidazole, similar to the study by Marais A. *et.al*. In this study mutations in RdxA were found much more than mutations in FdxA and FrxA, suggesting that the mutations in *rdxA* rather than *frxA* and *fdxA* may contribute to metronidazole resistance which was related to previous evidence from Yang YJ *et al*(46). Other mechanisms including efflux pump, DNA repair contributed to metronidazole resistance.

CHAPTER VII

CONCLUSION

Triple drug therapy that was standard therapy regimens for treating *Helicobacter pylori* infection, and used to combination with proton pump inhibitors (PPI). Antibiotics that are frequently included in triple therapy regimens are metronidazole, clarithromycin, tetracycline and metronidazole is an essential component for *H. pylori* eradication. However, recent reports suggest that metronidazole resistance is on the rise and is likely to become an increasingly important problem in the clinical management of *H. pylori* infection. Resistance to metronidazole (MTZ) in *Helicobacter pylori* is associated with mutations in *rdxA*, encoding an oxygen-insensitive NADPH nitroreductase, mutations in fdxA, encoding ferridoxin-like protein and mutations in *frxA*, encoding a NAD(P)H-flavin oxidoreductase. Despite this association, the strict correlation of MTZ resistance with mutations in *rdxA* or *frxA* is still unclear.

Thirty strains of metronidazole-resistant *Helicobacter pylori* and five strains of metronidazole-susceptible isolates were obtained from a collection culture stored at -70^{o}C the Department of Microbiology, Faculty of Medicine,King Chulalongkorn Memorial Hospital. The finding that *rdxA* sequence variations detected in high-level metronidazole-resistant isolates. Mutations in the *rdxA* gene were analyzed by PCR and DNA sequencing. The DNA sequences of *rdxA* were determined by sequencing both metronidazole–susceptible and metronidazole– resistant *H. pylori* isolates. Of the 35 isolates, 5 were susceptible to metronidazole (MICs range = < 0.016 – 8 μg/ml) and 30 isolate was resistant to metronidazole (MIC ≥ 8μg/ml). When compared with amino acid sequence of RdxA of *H. pylori* 26695 from GenBank accession no. AE000573, there have many different amino acid substitutions in both metronidazole-susceptible and -resistant isolates. There were 10-30 amino acid substitutions in RdxA among all isolates sequenced. Frameshift mutation was found in several metronidazole-resistant isolates, and majority leading to stop codon. Amino acid substitutions which caused prematured stop codon were also found in MTZ-resistant isolates. Amino acid substitutions were found in both MTZ-susceptible and MTZ-resistant isolates. In FdxA, amino acid changes in FdxA are shown in Table 15 and Appendix. Only found one of 35 *H. pylori* isolates,828, a metronidazole-resistant

isolate, contained a single missense mutation within the *fdxA* gene, Asn_{32} to His. Six metronidazole-resistant isolates had amino acid deletion at Asp_{47} that occurred by nucleotide TGA deletion at nucleotide position 138-140. All of 5 metronidazole-susceptible isolates not found the mutation in FdxA. We analyzed the FrxA amino-acid sequences to identify any amino-acid changes. Four of 5 metronidazole-susceptible *H. pylori* was found 1-4 amino acid substitutions. Truncated FrxA due to a frameshift mutation was found in 8 metronidazole-resistant strains. Amino acid substitution at position 7, 16, 111, 124 and 193 was found in both metronidazole-susceptible and -resistant isolates. In conclusion, from this study we found the mutation both in RdxA, FdxA and FrxA were 7(23.33%) of 30 metronidazole-resistant isolates, and was found the mutation in 2 proteins were 23(76.67%) of 30 metronidazole isolates, and all of metronidazole-resistant isolates were found the mutation at least in one of these nitroreductases(100%).

From this results we suggest that alterations in *rdxA*, *fdxA* and *frxA* genes and their upstream regions may be involved in the development of *H. pylori* resistance to metronidazole.

Therefore, mutation in RdxA, FdxA and FrxA may not be essential associated with metronidazole resistance in *H. pylori*. Other mechanisms must be involved in metronidazole resistance such as efflux pump mechanisms

For study of the efflux pump mechanism (HefA), no detection the decreasing of MIC level of metronidazole with CCCP which the efflux pump inhibitor. RT-PCR could not detect mRNA expression of nitroreductase genes and HefA in both susceptible and resistant isolates. A sensitive method, Real-time RT-PCR, is considered to be used for further investigation. Our results demonstrated that mutations in nitro reductase genes including *rdxA*, *frxA* and *fdxA* were associated with metronidazole resistance in *H. pylori*

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APPENDICES

APPENDIX A

REAGENTS AND INSTRUMENTS

REAGENTS

Absolute ethanol (Merck, Germany)
Agarose (Biorad, USA)
Boric acid (Sigma, USA)
dNTPs (Promega, USA)
EDTA (Amresco, USA)
Ethidium bromide (Amresco, USA)
NaCl (Merck, Germany)
Taq DNA Polymerase (Fermentas, USA)
Tris (Amresco, USA)
100 bp DNA ladder (Fermentas, USA)
100 bp plus DNA ladder (Fermentas, USA)
NaOH (Sigma, USA)
Brain heart infusion agar (Oxoid, England)
Ethidium bromide (Amresco, USA)
Horse serum (GibcoBRL, USA)
Miniral oil (Sigma, USA)
Columbia agar base (Oxoid, England)
NaCl (Merck, Germany)

MATERIALS

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

INSTRUMENTS

Anaerobic jar (BBL, USA)

Gas pack (Oxoid, England

Automatic pipette (Gilson, Lyon, France)

Camera Gel DocTM MZL (BIO-RAD, USA)

Incubator (Forma Scientific, USA)

Perkin Elmer GeneAmp PCR system 9600 (Perkin Elmer, USA)

Microcentrifuge (Eppendorf, USA)

Spectrophotometer (BIO-RAD, USA)

Water bath (Memmert, USA)

APPENDIX B

MEDIA AND ANTIBIOTIC SOLUTION PREPARATION

1. Columbia agar with 7 % sheep blood

Columbia agar base 39 g/L

Horse serum 70 ml/L

Sheep blood 70 ml/L

Distilled water 860 ml

The medium was sterillized by autoclaving at 121 ^{o}C, 15 pounds/inch^{2} pressure, for 15 minutes. The sterile medium was cooled to 45 ^{o}C to 50 ^{o}C. Add blood and horse serum after cooling base medium. Dispense 20 ml per petridish. Cool and store at 4 ^{o}C until used. Do not add any heat labile components (Sheep blood or antibiotic solutions) to
the sterillizer.

2. Columbia agar with 7 % Sheep blood and antibiotics

Columbia agar base 39 g/L

Horse serum 70 ml/L

Sheep blood 70 ml/L

Vancomycin (1 ml of stock) 10 mg/L

Trimethoprim (0.5 ml of stock) 5 mg/L

Cefsoludin (0.5 ml of stock) 5 mg/L

Amphotericin B (0.5 ml of stock) 5 mg/L

Distilled water 860 ml

The medium was sterillized by autoclaving at 121^{o}C, 15 pounds/inch2 pressure, for 15 minutes. The sterile medium was cooled to 45 ^{o}C to 50 ^{o}C. Add blood, horse serum and antibiotic solution after cooling base medium. Dispense 20 ml per petridish. Cool and store at 4^{o}C until used. Do not add any heat labile components (Sheep blood or antibiotic solutions) to
the sterillizer.

3. Antibiotic solution preparation

Vancomycin, final concentration 10 mg/L

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

Cefsoludin, final concentration 5 mg/L

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

Trimethoprim, final concentration 5 mg/L

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

Amphotericin, final concentration 5 mg/L

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

4. BHI broth

Suspend 30 grams in 1 litre of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121 ^{o}C for 15 minutes. Once the medium is prepared, store at 4 ^{o}C.

5. Brain heart infusion with 20 % glycerol

Brain heart infusion 37 g/L

Glycerol 200 ml

Distilled water 800 ml

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

6. Sterile saline solution

Sodium Chloride 8.5 g/L

Distilled water 1 L

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

APPENDIX C

REAGENTS PREPARATION

1. 10x Tris-borate buffer (TBE)

Tris base	108 g/L
Boric acid	55 g/L
0.5 M EDTA (pH 8.0)	40 ml

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

2. 0.5 M EDTA (pH 8.0)

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

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

3. 10x TE buffer

Tris	12.11 g/L
0.5 M EDTA	20 ml

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

4. 1.5 % Agarose gel

Agarose	0.6 g
1x TBE	40 ml

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

5. 6x Loading buffer 100 ml

Tris HCl	0.6 g
EDTA	1.68 g
SDS	0.5 g

Bromphenol Blue	0.1 g
Sucrose	40 g

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

Reagent for DNA Extraction

1.1 Protease K

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

1.2 Buffer AL (Ready to used)

1.3 Buffer AW1

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

1.4 Buffer AW2

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

1.5 Buffer AE (Ready to used)

Reagent for PCR product purification

Buffer PB (Ready to used)

Buffer PE

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

APPENDIX D

64 CODON ON DNA

One- and Three-Letter symbols for the amino acids

A	Ala	Alanine
B	Asx	Asparagine or aspartic acid
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Trytophan
Y	Tyr	Tyrosine
Z	Glx	Gln or Glu

The standard genetic code

First position (5'end)	Second position				**Third position (3' end)**
	U	C	A	G	
	UUU Phe	UCU Ser	UAU Tyr	UGU Cys	U
U	UUU Phe	UCC Ser	UAC Tyr	UGC Cys	C
	UUA Leu	UCA Ser	UAA Stop	UGA Stop	A
	UUG Leu	UCG Ser	UAG Stop	UGG Trp	G
	CUU Leu	CCU Pro	CAU His	CGU Arg	U
	CUC Leu	CCC Pro	CAC His	CGC Arg	C
C	CUA Leu	CCA Pro	CAA Gln	CGA Arg	A
	CUG Leu	CCG Pro	CAG Gln	CGG Arg	G
	AUU Ile	ACU Thr	AAU Asn	AGU Ser	U
	AUC Ile	ACC Thr	AAC Asn	AGC Ser	C
A	AUA Ile	ACA Thr	AAA Lys	AGA Arg	A
	AUG Met[a]	ACG Thr	AAG Lys	AGG Arg	G
	GUU Val	GCU Ala	GAU Asp	GGU Gly	U
	GUC Val	GCC Ala	GAC Asp	GGC Gly	C
G	GUA Val	GCA Ala	GAA Glu	GGA Gly	A
	GUG Val	GCG Ala	GAG Glu	GGG Gly	G

[a]AUG forms part of the initiation signal as well as coding for internal Met residues.

Appendix E

Figure15. Multiple nucleotide sequence alignment of entire *rdxA* gene from 30 metronidazole-resistant isolates with those from 26695 (GenBank accession no. AE000511)

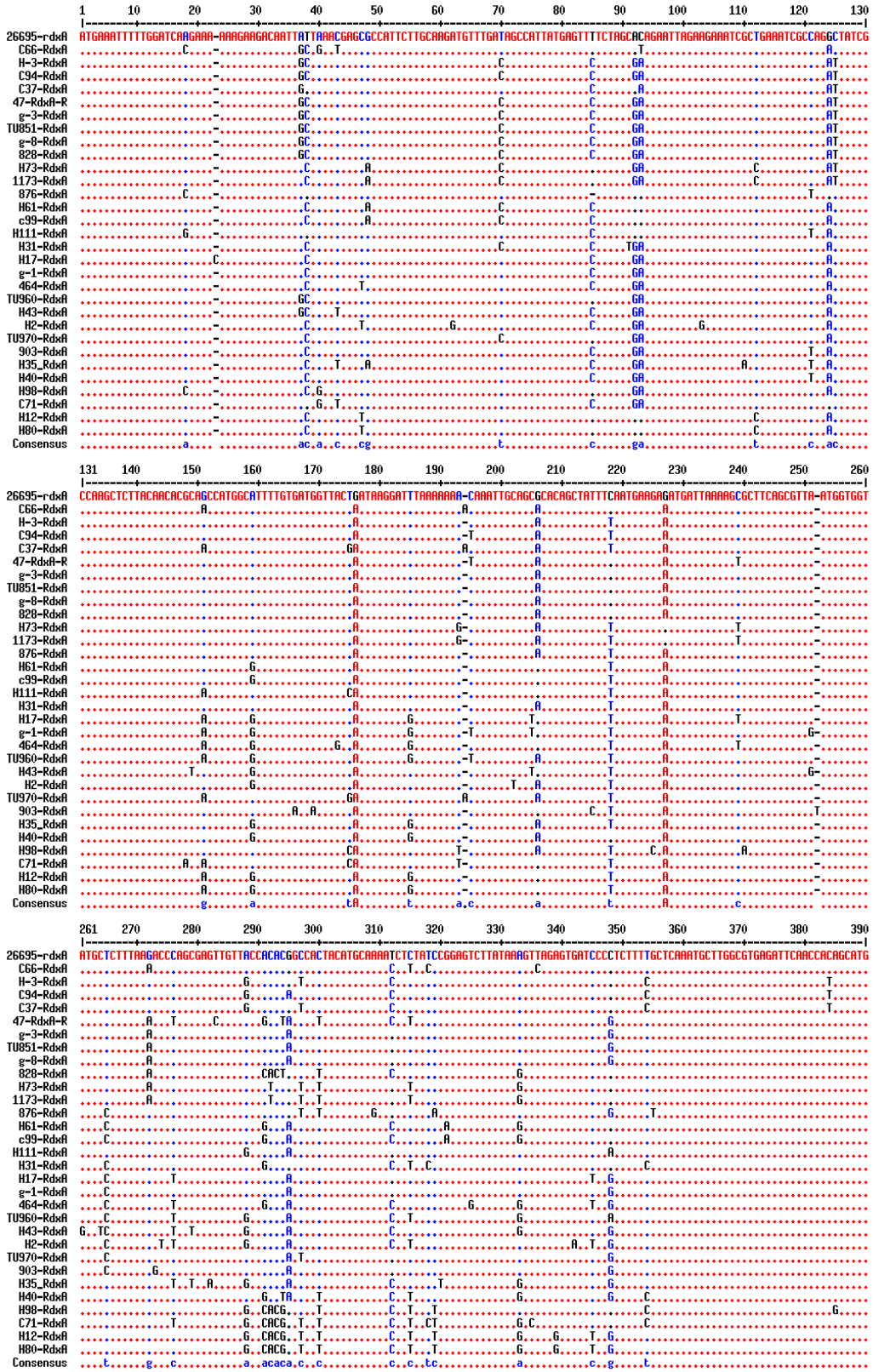


Figure15. (continued)

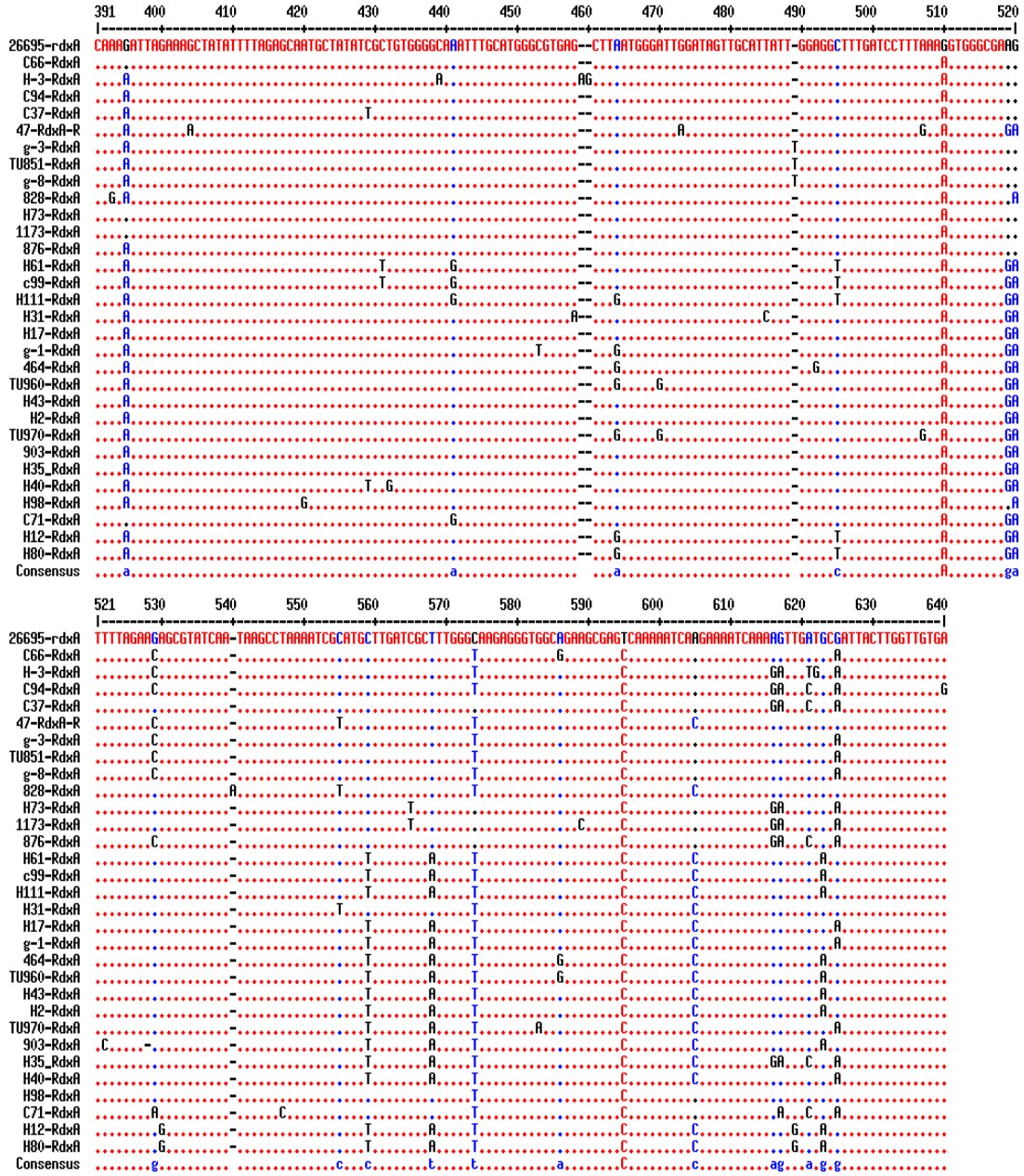


Figure16. Multiple nucleotide sequence alignment of entire *rdxA* gene from 5 metronidazole-susceptible isolates with those from 26695 (GenBank accession no. AE000511)

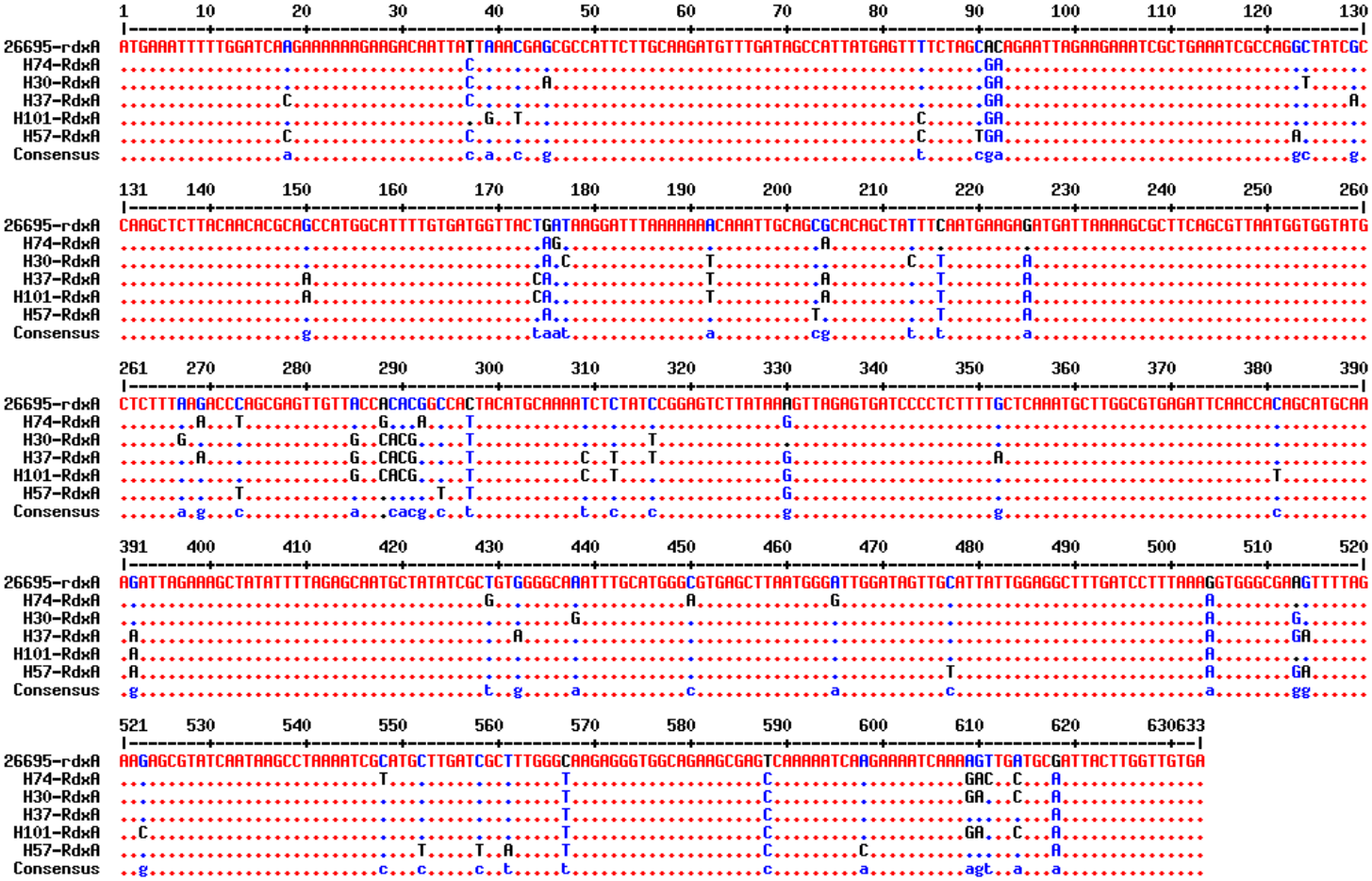


Figure 17. Multiple nucleotide sequence alignment of entire *fdxA* gene from 30 metronidazole-resistant isolates with those from 26695 (GenBank accession no. AE000511)

Figure 18. Multiple nucleotide sequence alignment of entire *fdxA* gene from 5 metronidazole-susceptible isolates with those from 26695 (GenBank accession no. AE000511)

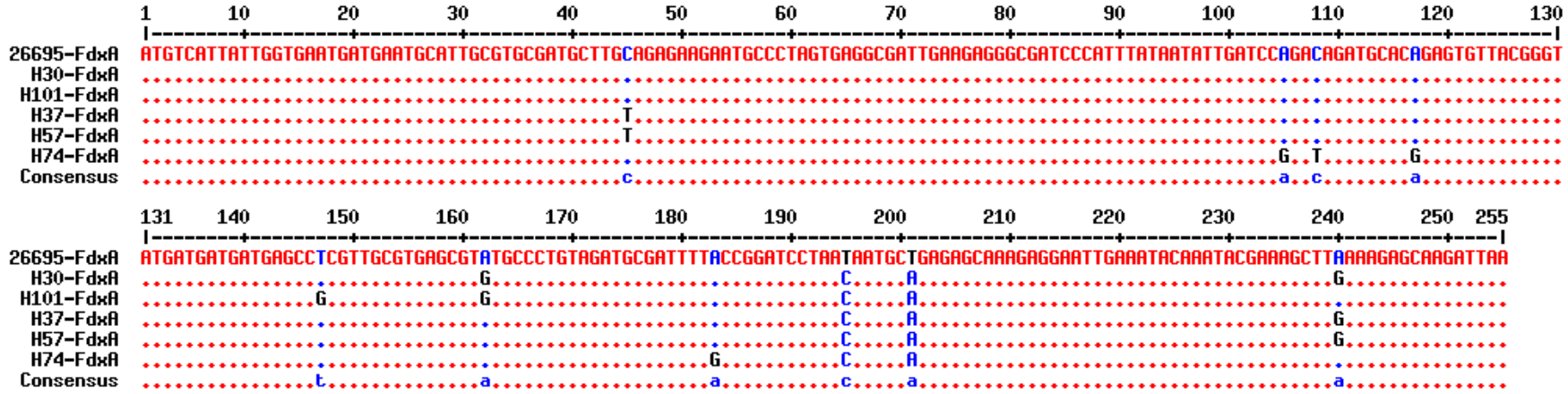


Figure 19. Multiple nucleotide sequence alignment of entire *frxA* gene from 30 metronidazole-resistant isolates with those from 26695 (GenBank accession no. AE000511)

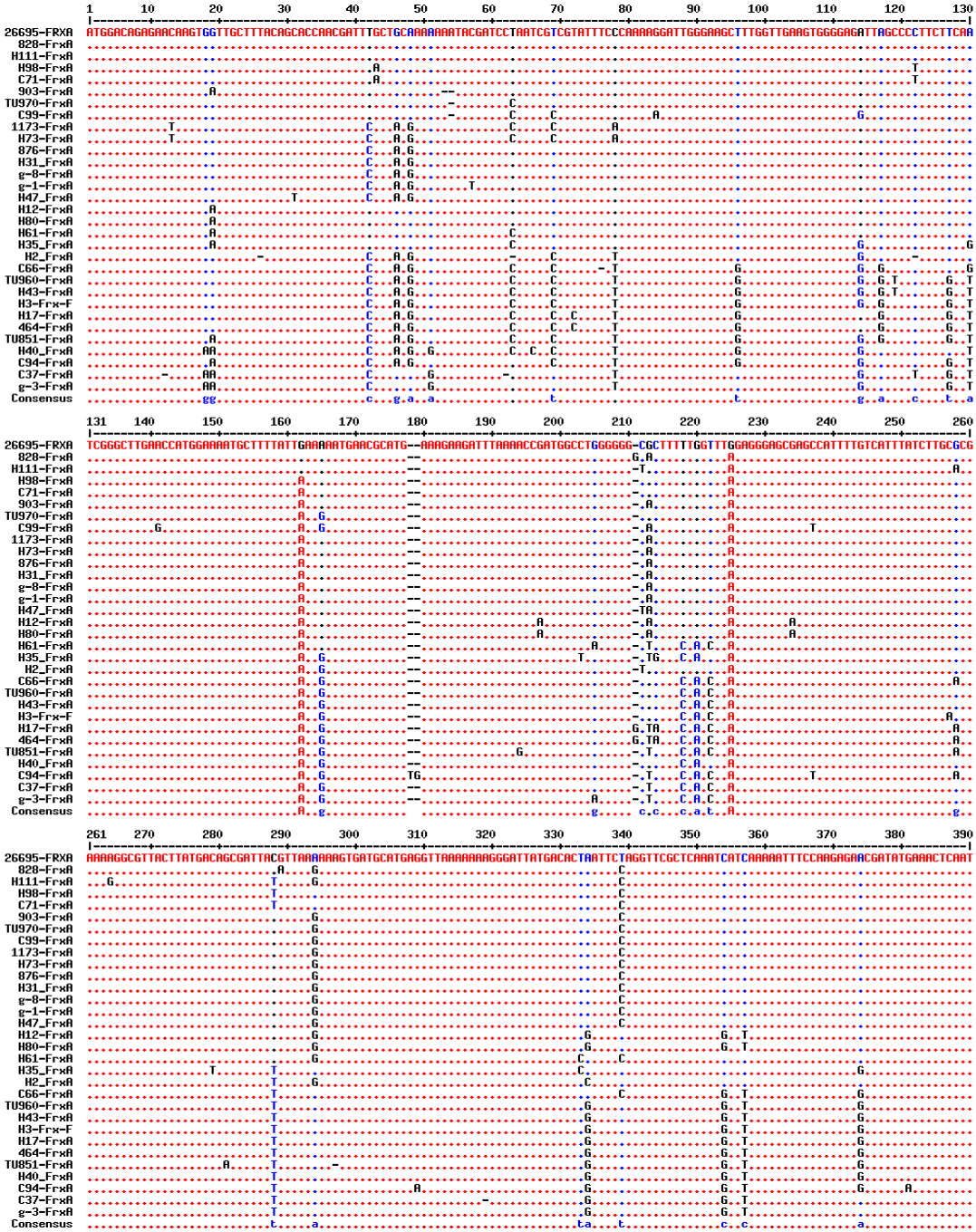


Figure 19. (continued)

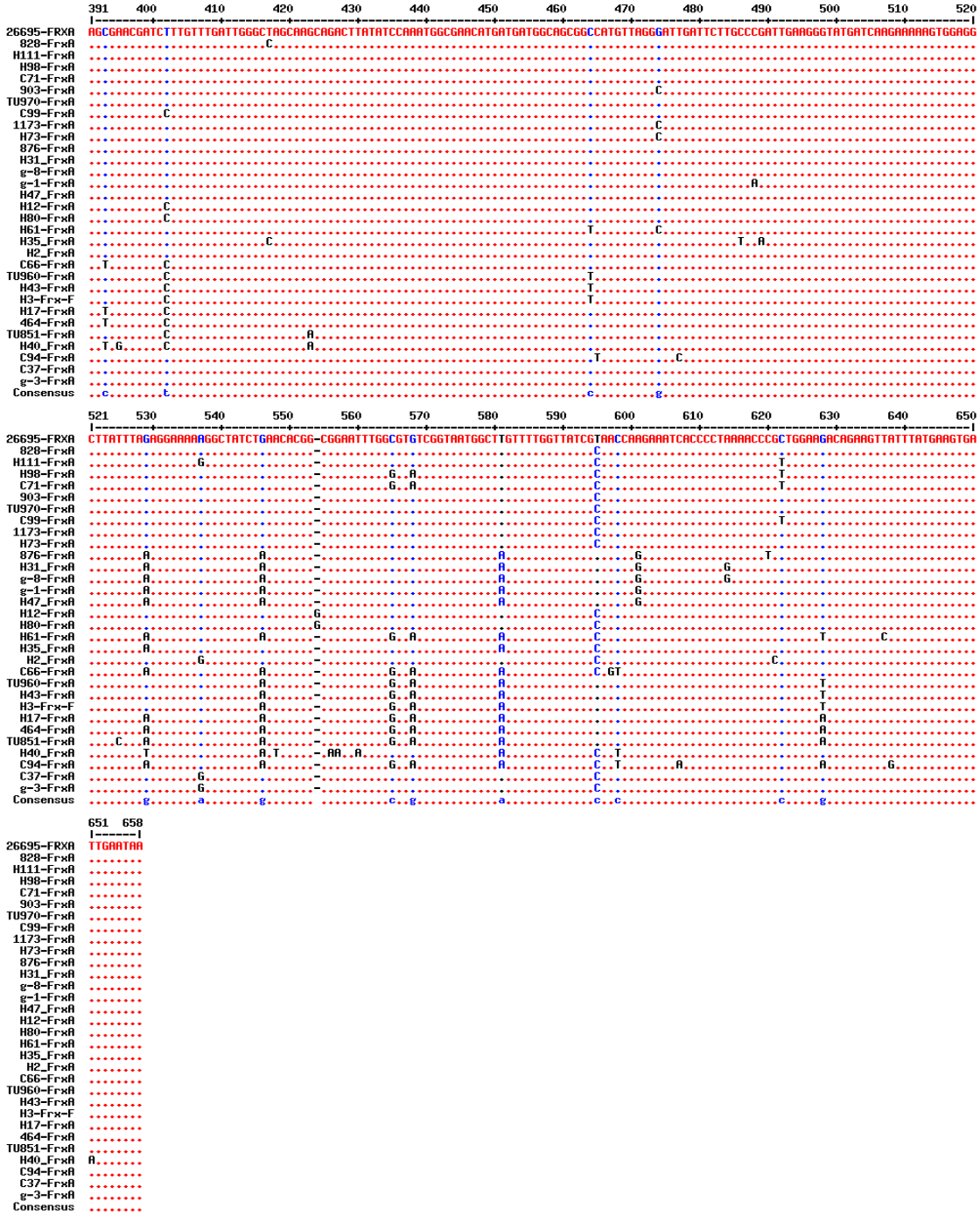


Figure 20. Multiple nucleotide sequence alignment of entire *frxA* gene from 5 metronidazole-susceptible isolates with those from 26695 (GenBank accession no. AE000511)

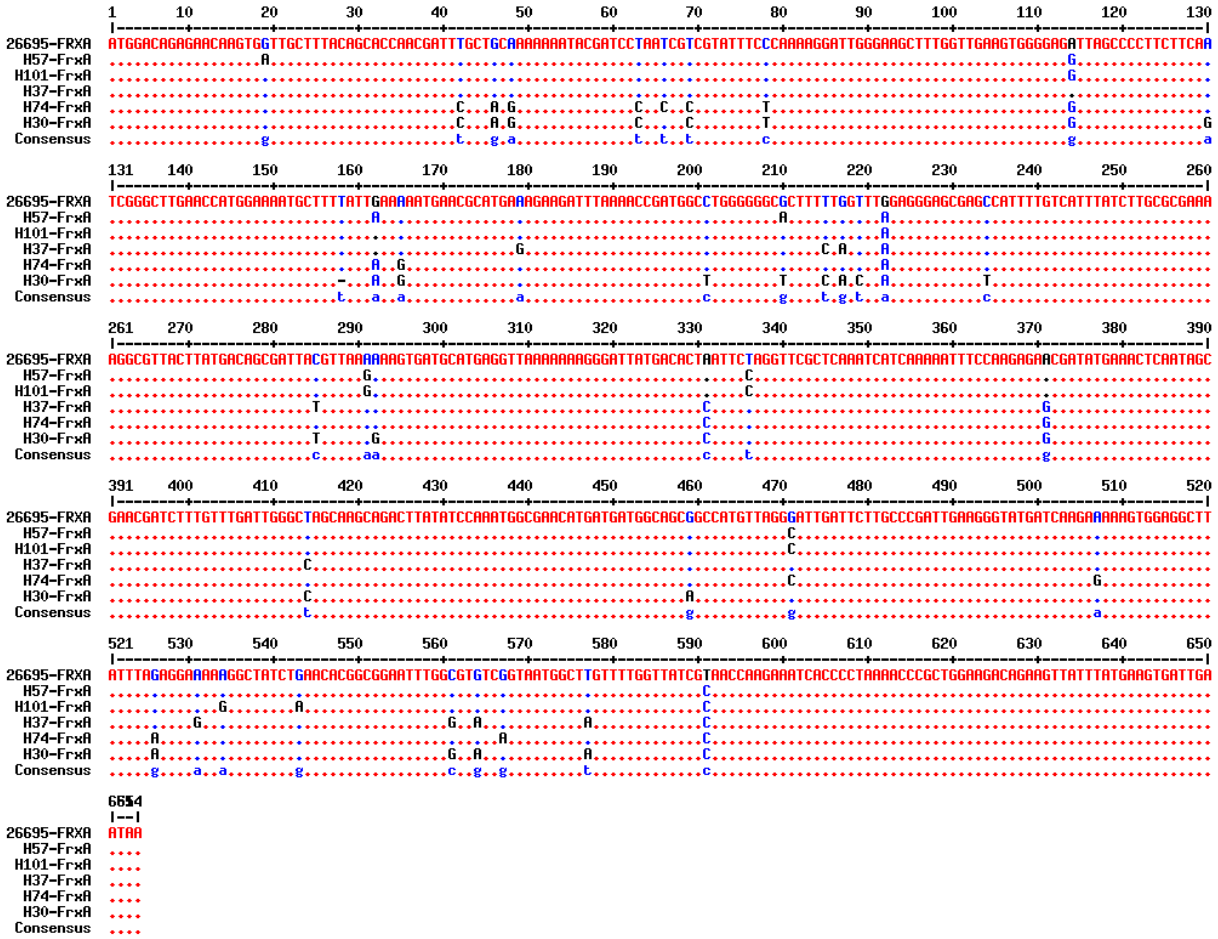


Figure 21. Multiple amino acid sequence alignment of entire RdxA protein from 30 metronidazole-resistant isolates with those from 26695 (GenBank accession no. AAD07703)

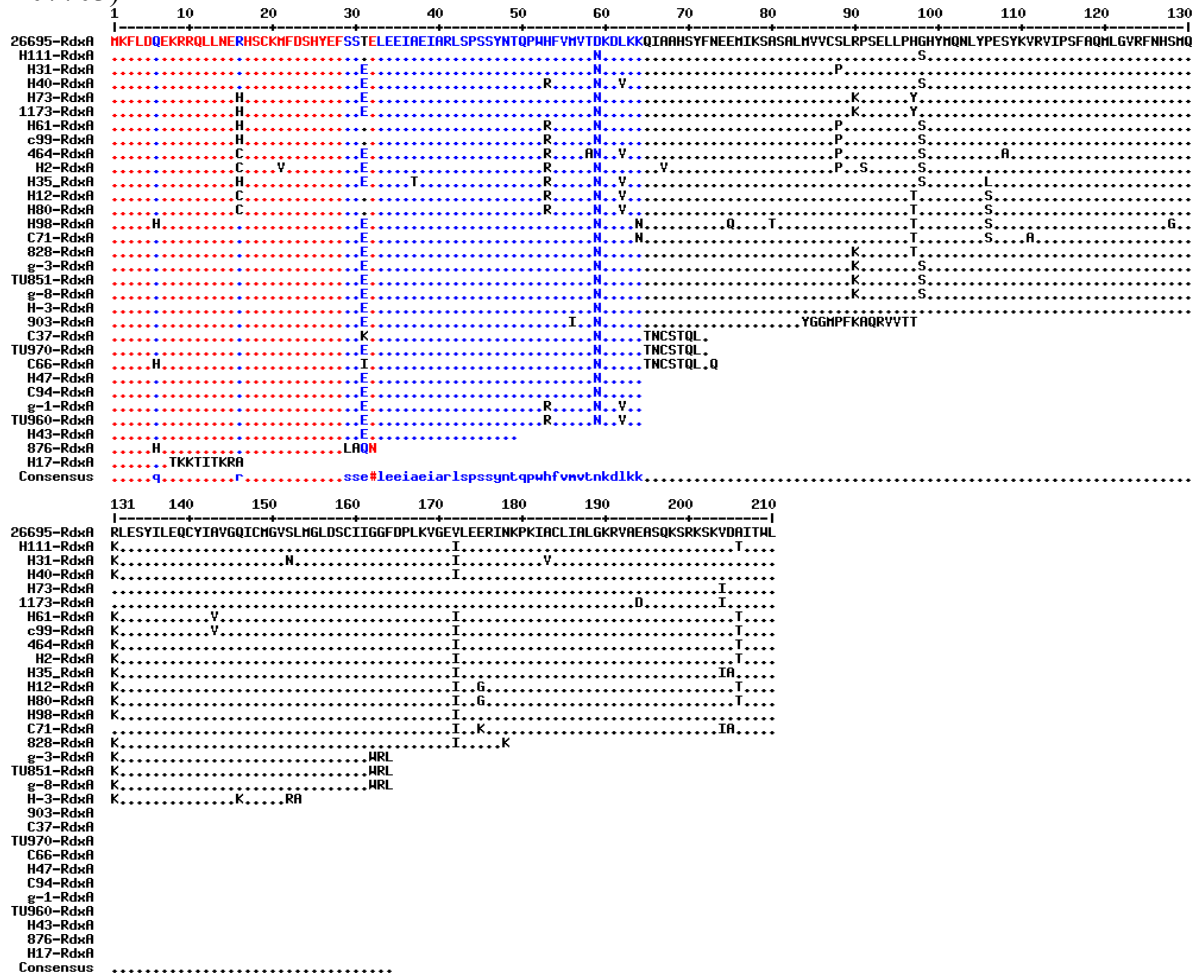


Figure 22. Multiple amino acid sequence alignment of entire FdxA protein from 30 metronidazole-resistant isolates with those from 26695 (GenBank accession no. AAD07340)

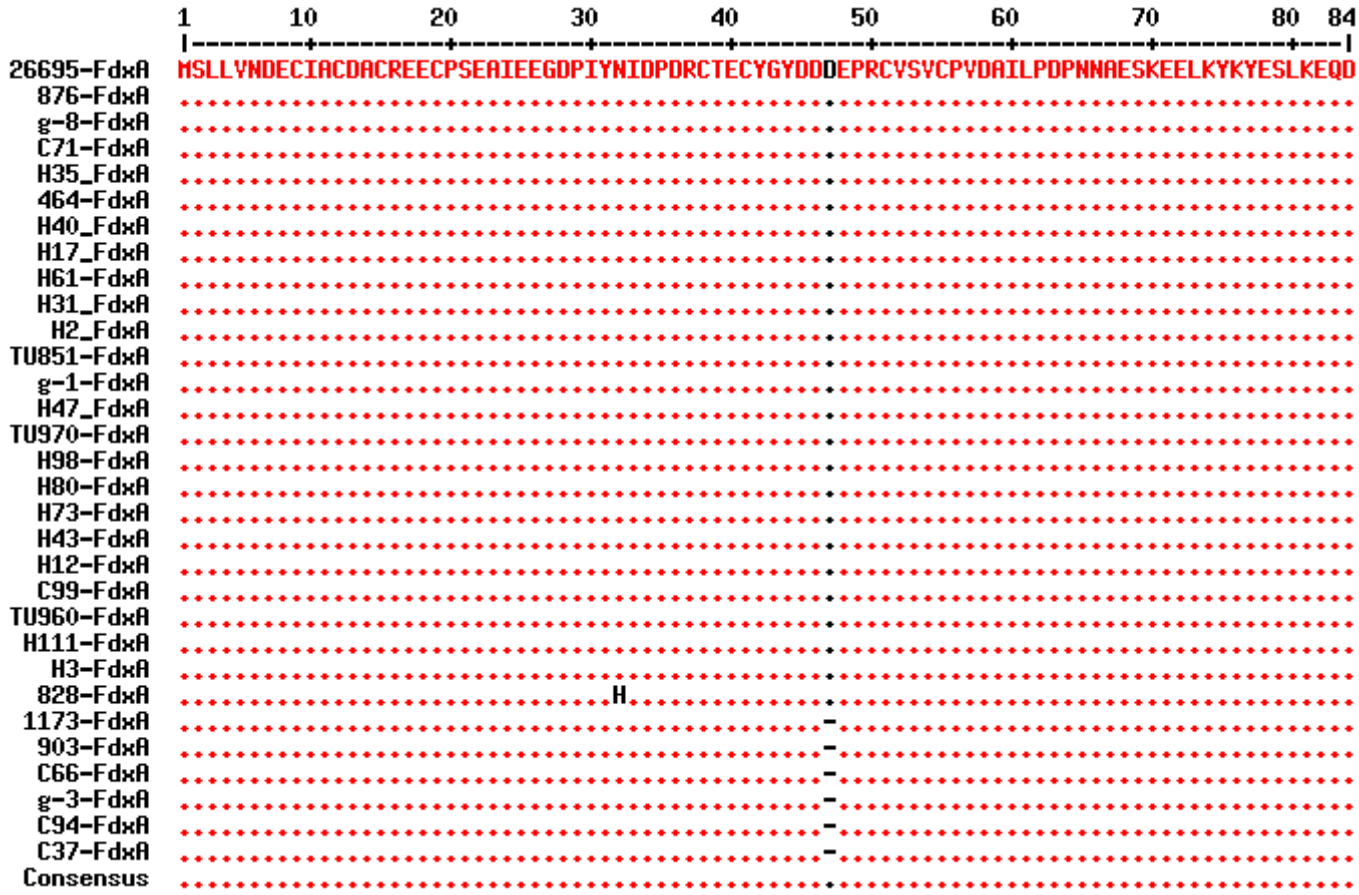
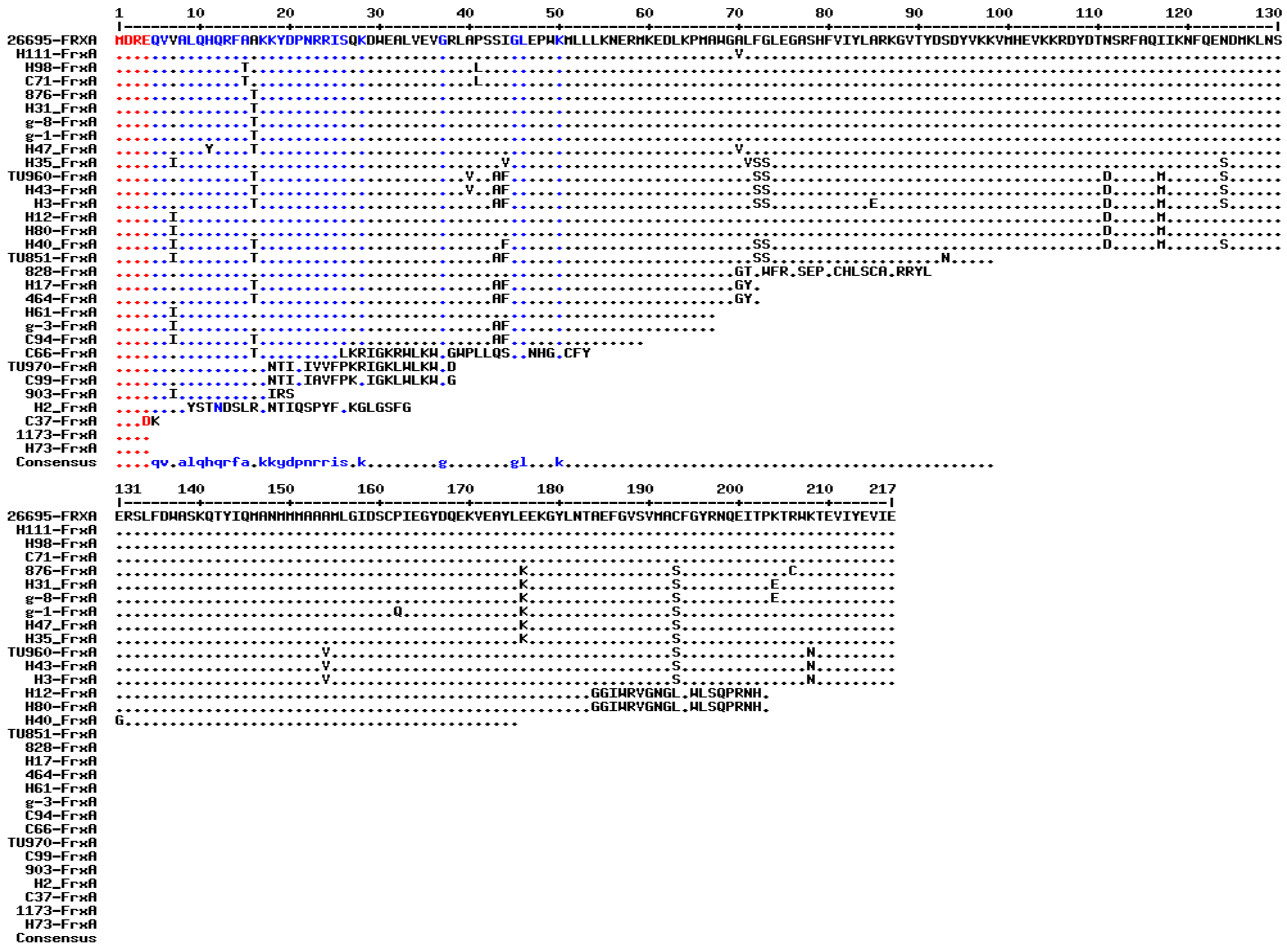


Figure 23. Multiple amino acid sequence alignment of entire FrxA protein from 30 metronidazole-resistant isolates with those from 26695 (GenBank accession no. AAD07703)



BIOGRAPHY

Miss Ornsiri Chueain was born on July 31, 1984 in Bangkok, Thailand. She graduated with the Bachelor degree of Science (Microbiology) from the Faculty of Sciences, Srinakharinwirot University in 2005. She is currently a student in the Inter-Department of Medical Microbiology, Faculty of Graduate School, Chulalongkorn University since 2010.