# Antibiotic Resistance in *Helicobacter pylori*


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# Abstract

*Helicobacter pylori* is a gram negative, microaerophilic bacteria that plays an important role in chronic gastritis and peptic ulcer disease. Multiple antimicrobial therapies including combinations of clarithromycin, metronidazole or amoxycillin and proton pump inhibitor have been used to eradicate *H. pylori*. Antimicrobial resistance in *H. pylori* has been associated with treatment failure. In this study, antimicrobial susceptibility patterns of amoxycillin, ciprofloxacin, clarithromycin, erythromycin, tetracycline and metronidazole were evaluated in 110 *H. pylori* strains isolated from 454 antral biopsies of patients undergoing endoscopy at the Royal Infirmary, Edinburgh. The MICs were determined by E-test. Resistance to clarithromycin and erythromycin was found in 8.2% (9/110) and 9.1% (10/110) respectively. Metronidazole resistance was demonstrated in 8 isolates (7.3%). Tetracycline resistance was found in one of the isolates (0.9%). Two isolates were resistant to ciprofloxacin (1.8%). Resistance to amoxycillin was not detected.

Molecular mechanisms of fluoroquinolone, macrolide and metronidazole resistance in *H. pylori* were investigated. Resistant to fluoroquinolone has been associated with alterations in the Quinolone Resistance-Determining Region (QRDR) of *gyrA* gene. Mutation at position 91, leading to an amino acid change from Aspartic acid to Asparagine was found in 2 ciprofloxacin-resistant isolates. One isolate had a mutation at Asparagine-87 to Lysine. Mutations in the 23S rRNA conferring macrolide resistance were investigated. Mutations at position 2143 (A to G) were shown in seven of the ten macrolide-resistant isolates. Two of the seven isolates

carried an additional T to C mutation at either position 2182 or 1934. Of the ten macrolide-resistant isolates, two had a single mutation at either position 2182 or 2195. Mutation at position 2182, however, has previously been identified not to be associated with macrolide resistance. The mutations at position at 1934 (T to C) and position 2195 (C to T) have not previously been reported. One of the ten isolates (MIC>256 mg/L) had no alteration in the 23S rRNA. The results indicate that different mechanisms play a role in macrolide resistance in these *H. pylori* strains. Metronidazole resistance has been reported to be associated with mutations in the *rdxA* gene, encoding oxygen-insensitive nitroreductase. To investigate the role of *rdxA*, sequencing analysis of *rdxA* of metronidazole-resistant isolates was determined. The results showed that no particular amino acid substitution was associated with metronidazole resistance. One isolate contained a nonsense mutation, generating a stop codon. However, two metronidazole-sensitive strains had alterations in *rdxA* by insertions of a mini-IS*605* sequence. These results suggest that alterations in *rdxA* are not the sole mechanism of metronidazole resistance and other mechanisms are required in the development of resistance.

Since the prevalence rate of metronidazole resistance is variable, ranging from 11 to 70%, it is possible that some variation in reported resistance levels derives from difficulties in the method of sensitivity testing. To set a standard for susceptibility testing for metronidazole in *H. pylori*, the optimum conditions for sensitivity testing were evaluated. Activation of metronidazole requires an anaerobic environment. It was found that incubation under microaerophilic conditions elevated metronidazole MIC, suggesting that microaerophilic conditions cannot activate metronidazole to its

active form. Pre-incubation of *H. pylori* in anaerobic conditions for 24 hours prior to incubation under microaerophilic conditions for 72 hours was found to be necessary to achieve accurate susceptibility results. This can explain why some centres report high levels of metronidazole resistance.

To investigate the development of fluoroquinolone resistance in *H. pylori*, ciprofloxacin-resistant mutants were selected *in vitro* by exposing sensitive strains to serial increments of ciprofloxacin in Columbia blood agar plate. The QRDR of *gyrA* gene was analysed for mutations. Reduced susceptibility to ciprofloxacin was associated with either a single or double amino acid changes in *gyrA* gene. Mutations at position 85, 87 and 91 were found to be associated with ciprofloxacin resistance. The results also demonstrated that *gyrA* mutations are not sole contributors for the mechanism of ciprofloxacin resistance in *H. pylori*. As no *parC* gene has not yet been identified in *H. pylori*, any contribution from topoisomerase IV cannot be quantified. To investigate the DNA gyrase activity in *H. pylori*, *gyrA* and *gyrB* were separately cloned and overexpressed by using a T7 promoter vector, which contain a fusion tag of six histidine residues. GyrA and GyrB were purified by affinity chromatography using nickel-chelating resins. The ability of DNA gyrase to supercoil relaxed DNA was determined.

# Declaration

The experiments and composition of this thesis are the work of the author unless otherwise stated.

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# Publication and Presentations

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# Abbreviations

| | |
|---|---|
| bp | base pairs |
| DNA | deoxyribonucleic acid |
| dNTP | deoxynucleotide triphosphate |
| E-test | Epsilon test |
| Kb | kilo base |
| kDa | kilo Daltons |
| L | litre |
| MIC | Minimum Inhibitory Concentration |
| M | molar |
| mM | milli molar |
| ml | milli litre |
| mg | milli gram |
| µg | micro gram |
| µl | micro litre |
| µm | micro metre |
| min | minutes |
| NCTC | National Collection of Type Cultures |
| OMP | Outer membrane protein |
| PCR | Polymerase Chain Reaction |
| QRDR | Quinolone Resistance Determining Region |
| s | seconds |
| SDS | Sodium Dodecyl Sulphate |

| | |
|---|---|
| TAE | Tris-acetate buffer |
| UV | Ultraviolet |
| V | Volts |

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# Chapter 1 Introduction

## 1.1 Overview

Since *Helicobacter pylori* was first isolated in 1982 by Marshall and Warren (Marshall and Warren, 1984), it has been recognized as the principal cause of peptic ulcer disease and as a risk factor in the development of gastric cancer (Parsonnet *et al.*, 1991). The discovery of the role of *H. pylori* infection has led to a breakthrough in medical care. Successful eradication of *H. pylori* infection requires a combination of two or three antibiotics and proton pump inhibitors. However, antibiotic resistance has emerged and is a principal cause of treatment failure. Understanding the mechanism of resistance will lead to better antibiotic administration and may lead to a design of novel and more effective drugs for a new successful treatment of *H. pylori*. Furthermore, identification and characterization of resistant gene will provide a sensitive and rapid method to detect antibiotic-resistant strains.

## 1.2 Discovery of *H. pylori*

The discovery of these bacteria was revealed by a series of unrelated observations. These were not obvious indicators at the time but are now clear demonstration of *H. pylori*. In 1893, Bizzozero first described the presence of spiral-shaped bacteria in the gastric glands and parietal cells of dogs. Krienitz, in 1906, noted that gastric spirochaetes were found in human stomach. In 1938, Doenges reported spirochaetes

present in 43% of 242 samples from autopsy stomachs. In 1975, spiral bacteria were demonstrated in 80% of gastric samples from patients with gastric ulceration (Steer, 1975). In 1979, Fung *et al.* described endoscopic, histological and ultrastructural correlations of chronic gastritis and showed that spiral bacteria were on the lumen surface of epithelial cells (Fung *et al.*, 1979).

It was almost one century after Bizzozero described spiral-shaped bacteria that Warren and Marshall, in 1982, were the first able to culture spiral organisms from gastric biopsies obtained from patients with upper gastrointestinal complaints. They used methods for the isolation of campylobacter species and incubated the cultures under microaerophilic conditions for 48 hours. The initial cultures were unsuccessful. During the Easter weekend, the plates were unintentionally incubated for 5 days and colonies were seen. Subsequently, other biopsy samples were also cultured for 3-4 days and the organisms were isolated. These organisms were characterized and called *Campylobacter pyloridis* since they were resembled other campylobacter species in morphological characteristics and guanine/cytosine content (Marshall and Warren, 1984). Subsequently, the species name was changed to *C. pylori*. In 1989, Goodwin suggested that *C. pylori* should be transferred to the new genus *Helicobacter* on the basis of comparison of 16S rRNA sequences, fatty acid contents, biochemical reactions and ultrastructure features (Goodwin *et al*, 1989). Following publication by Marshall and Warren (1984), the presence of these organisms in the gastric mucus was rapidly confirmed (Jones *et al.*, 1984; McNulty and Watson, 1984).

In an effort to prove that these curved bacteria were the cause of upper gastrointestinal disease, Marshall conducted an ingestion study by infecting himself and developed gastritis afterwards (Marshall *et al.*, 1985). Koch's postulates were, therefore, fulfilled and the association between *H. pylori* and upper gastrointestinal disease has been established. Since the discovery of *H. pylori*, the perception of peptic ulcer disease has subsequently altered from chronic, recurring disease of uncertain origin to a disease, caused by bacteria, which can be cured by antibiotic-based treatments.

In 1994, the International Agency for Cancer Research stated that *H. pylori* was a grade I carcinogen of humans and a consensus conference convened by the National Institutes of Health concluded that *H. pylori* was a major cause of peptic ulcer disease and strongly recommended that patients with *H. pylori* infection and gastric or duodenal ulcers should be treated to eradicate the bacterium (NIH Consensus Conference, 1994).

*H. pylori* infection also has been associated with the development of gastric non-Hodgkin's lymphomas (Parsonnet *et al.*, 1991) and gastric mucosa-associated lymphoid tissue (MALT) lymphoma (Eidt *et al.*, 1994; Wotherspoon *et al.*, 1991). *H. pylori*, therefore, has now been linked to many diseases associated with gastroduodenal tissue.

## 1.3 Microbiological Characteristics

*H. pylori* organisms are curved to spiral-shaped, Gram-negative bacteria that colonise the gastric lumen of primates, including human. After prolonged culture, coccoid forms typically predominate (Bode *et al.*, 1993) and are metabolically active but cannot be cultured *in vitro*. *H. pylori* is found to be 2.5 to 5.0 μm long and 0.5 to 1.0 μm wide with four to six unipolar sheathed flagella in gastric biopsy samples (Goodwin and Armstrong, 1990; Goodwin *et al.*, 1985). Flagella are important for bacterial motility. *H. pylori* are mostly actively motile but may appear to be non-motile in hanging drop preparations in some cultures (Owen, 1998). *H. pylori* are microaerophilic bacteria, growing best in an atmosphere of 5% oxygen, however, after laboratory passages, some strains can grow in 10% $CO_2$ (Goodwin and Armstrong, 1990). *H. pylori* requires complex basal medium with supplementation such as whole blood, serum, haem, cornstarch, charcoal, or egg yolk emulsion (Hachem *et al.*, 1995; Henriksen *et al.*, 1995). Colonies of *H. pylori* with circular (1-2mm), convex and translucent on blood agar normally take 3-5 days at 37°C to appear for the primary isolation. *H. pylori* does not oxidise or ferment carbohydrates. It produces catalase, oxidase and high level of urease and alkaline phosphatase (Owen, 1998).

The genome size of *H. pylori* ranges from 1.6 to 1.73 Mb, with an average of 1.67 Mb (Taylor *et al.*, 1992). The G+C content ranges from 34.1 to 37.5mol% with an average value of 35.2 mol% (Beji *et al.*, 1988). *H. pylori* exhibits considerable re-arrangements in gene order and sequence variation within gene (Jiang *et al.*, 1996), including genes

that encode urease (Foxall *et al.*, 1992) and accessory proteins, flagellin (Forbes *et al.*, 1995), vacuolating cytotoxin (VacA) (Cover *et al.*, 1994), and Cytotoxin-associated protein (CagA) (Garner and Cover, 1995; Kato *et al.*, 1996; Miehlke *et al.*, 1996). Although, plasmids are present in about 40% of *H. pylori* strains with the size ranging from 1.5 to 23.3 kb, they do not have known virulence factors (Kleanthous *et al.*, 1991; Minnis *et al.*, 1995). The lipopolysaccharide (LPS) expresses Lewis blood group antigens (Appelmelk *et al.*, 1997), which may play a role in pathogenesis.

## 1.4 *H. pylori* Infection

*H. pylori* colonise the stomachs and cause continuous inflammation (Dooley *et al.*, 1989), leading to increasing risk for a wide range of clinical outcomes. *H. pylori* infection is recognised as a cause of chronic active gastritis and peptic ulcer disease. It is considered to be a risk factor for gastric cancer and MALT lymphoma (Parsonnet *et al.*, 1991; Parsonnet *et al.*, 1994; Peterson, 1991). The majority of infected persons do not develop clinically apparent disease. It is likely that a combination of several factors, such as strain pathogenicity, environmental conditions and individual host response influences the disease outcome (Cremonini *et al.*, 2001).

*H. pylori* produces a variety of substances and enzymes that cause injury to the epithelial cells, such as urease (Labigne *et al.*, 1991), superoxide dismutase (Spiegelhalder *et al.*, 1993) and phospholipases (Mauch *et al.*, 1993). Vacuolating cytotoxin (VacA) and cytotoxin-associated protein (CagA) are produced by only some clinical isolates

(Covacci *et al.*, 1993; Cover *et al.*, 1995; Cover *et al.*, 1994; Phadnis *et al.*, 1994). Although *vacA* is present in nearly all *H. pylori* strains, only 40-60% of the strains have cytotoxin activity (Cover *et al.*, 1990; Weel *et al.*, 1996). The *vacA* gene type s1 is associated with enhanced gastric inflammation and more severe diseases (Atherton *et al.*, 1997). *H. pylori* strains (60%) carrying the *cagA* gene have been reported to be associated with enhanced expression of interleukin-1α (IL–1 α ), interleukin-1β (IL-1β) and interleukin-8 (IL-8) and mucosal inflammation (Peek *et al.*, 1995). The release of IL-8, which has potent chemotactic activity for neutrophils, plays a role in gastric inflammation and cell damage (Censini *et al.*, 1996). There is a close association between the presence of *cagA* and *vacA* type s1. Most s1 strains are *cagA* positive (Atherton *et al.*, 1995). Autoimmunity may play a role in epithelial cell destruction and mucosal damage as Lewis blood antigen has been found to be expressed on the lipopolysaccharide of *H. pylori* (Appelmelk *et al.*, 1996).

## 1.5 Epidemiology of *H. pylori* Infection

*H. pylori* is one of the commonest bacteria in humans. It has been found in the stomachs of humans in all parts of the world. In developing countries, 70 to 90% of the population carries *H. pylori*. The prevalence of infection is lower in developed countries, ranging from 25 to 50%. The acquisition seems to occur predominantly in childhood (Goodman and Correa, 1995; Mendall and Northfield, 1995).

Most studies suggest that males and females have the same risk of becoming infected in all age groups considered. In developed countries, *H. pylori* infection rate is lower in individuals with higher socioeconomic status although the rate of infection is high among certain ethnic groups despite economic advancement (Graham *et al.*, 1991; Mendall *et al.*, 1992)

### 1.5.1 Transmission

Since *H. pylori* is a bacterium restricted to humans and other primates, its route of transmission remains unclear. Various pathways have been discussed, favoring a person-to-person mode of transmission. It is suggested that both fecal-oral and oral-oral transmission is predominant in childhood (Thomas, 1994). In particular, *H. pylori* has been isolated from the faeces of infected children (Thomas *et al.*, 1992), indicating that shedding is intermittent. Faecally contaminated water may be a source of infection (Klein *et al.*, 1991). *H. pylori* can reach the oral cavity. Oral-oral transmission has been

reported in the case of African women who premasticated foods given to their infants (Megraud, 1995). Sexually transmission has not been identified (Perez-Perez *et al.*, 1991; Polish *et al.*, 1991).

Childhood is thought to be the primary period of risk for *H. pylori* acquisition (Malaty and Graham 1994). *H. pylori* can be found in saliva, dental plaque (Ferguson *et al.*, 1993; Krajden *et al.*, 1989) and faeces (Leverstein-van Hall *et al.*, 1993) which could be the vehicle of transmission through anything contaminated by them.

## 1.6 Diagnosis of Infection

Many diagnostic tests are available for the diagnosis of *H. pylori* infection including invasive methods requiring endoscopy and biopsy and non-invasive methods (Table 1.1). Non-invasive tests for the diagnosis of *H. pylori* infection are widely used in clinical practice.

**Table 1.1 Diagnostic tests for detection of *H. pylori* infection**

| Invasive method | Noninvasive methods |
|---|---|
| Culture | Serology |
| Histology | Urea breath test |
| Rapid urease test | Stool antigen test |
| Molecular biology techniques such as PCR | |

## 1.6.1 Invasive Test

### 1.6.1.1 Culture

*H. pylori* culture is definite proof of infection. However, it is the most technically demanding of the diagnostic techniques. The optimum conditions of isolation, transportation and culture media are required. The sensitivity of culture in experience laboratories is greater than 90% (Megraud *et al.*, 1999) but it can vary extensively between centres because of local expertise. The lengthy incubation period is also required for *H. pylori* culture (3-12 days). Although considered a tedious, time-consuming, and expensive procedure, culture is the standard technique used in most laboratories for the isolation of *H. pylori* from gastric biopsy samples. The advantage of culture is that it allows the determination of antimicrobial susceptibility testing and the strains can be analysed in detail. The antibiotic susceptibility profiles are needed particularly after treatment failure. However, the standard protocol for *H. pylori*

susceptibility testing has not yet been established. It also demands money, time and personnel and is mainly used in research centres.

Culture of gastric biopsy samples provides the best yield of *H. pylori*. Biopsies from the antrum are mainly used since *H. pylori* usually colonises at this site. Transport has to be rapid and the transport media such as normal saline, 20% glucose or Stuart's medium are required (Soltesz *et al.*, 1992). Saline and 20% glucose are a simple and acceptable for a short time period (<4 hours) and should be maintained at 4°C. For a longer period (24 hours), Stuart's medium must be used and maintained at 4°C. The biopsy samples can be kept at –70°C or in liquid nitrogen (Han *et al.*, 1995).

Many different media, such as Dent's medium and Skirrow's medium, can be used to grow *H. pylori*. It mainly consists of an agar base such as Columbia or brain heart infusion agar, a growth supplement such as horse blood or serum and a selective supplement, including a mixture of antimicrobial agents such as vancomycin, trimethoprim, cefsulodin and amphotericin B, which prevents the growth of other organisms. The use of both selective and non-selective media may increase sensitivity.

*H. pylori* requires a microaerophilic environment with high humidity. The incubation time should be for a maximum of 7-10 days at 37°C. Positive cultures are usually observed three to five days after plating the biopsies. The small, round, smooth and translucent colonies on the selective media can be identified as *H. pylori*. Gram-strain

(Gram-negative, curved rods) and biochemical tests, including urease, catalase and oxidase, which give strong positive results, should be carried out to complete the identification.

### 1.6.1.2 Histology

Several staining method, such as modified Geimsa and Warthin-Starry, are used to detect *H. pylori*. In experienced hands, histological identification is highly sensitive and specific (Cutler *et al.*, 1995) and provides the assessment of morphological changes of the gastric mucosa. Immunohistochemistry, which is a sensitive and specific staining method, has been established to detect *H. pylori* (Ashton-Key *et al.*, 1996). The sensitivity of the histology is generally 90-95% and the specificity is 95-98% (Nedenskov-Sorensen *et al.*, 1991). Factors affecting the capability of test include bacterial density, type of stain used, and the expertise and enthusiasm of the pathologist.

### 1.6.1.3 Rapid Urease Test

Since *H. pylori* produces large amount of urease enzyme, the indirect detection of the organism has been developed. The sensitivity of the tests depends on the number of bacteria in the biopsy. There are different types of rapid urease tests commercially available, including the gel test (CLOtest, Hpfast), paper tests (PyloriTex®) and tablet test. In pre-treatment, the sensitivity of the tests varies from 80-95% whereas the specificity range is 95-100% (Midolo and Marshall, 2000). In post-treatment and in

bleeding patients, it has been reported the low sensitivity and specificity (Archimandritis *et al.*, 2000; Tu *et al.*, 1999). The CLOtest was the first commercial available urease test for biopsy samples. It consists of an agar gel containing phenol red and urea. In the presence of urease, the urea is hydrolysed and forms ammonia, leading to an increase in pH, which is detected by the change of the indicator, phenol red, from yellow to red. The test is interpreted up to 24 hours.

#### 6.1.1.4 PCR

Polymerase chain reaction (PCR) can be used for the detection of *H. pylori* in gastric tissue samples. It is a rapid and highly sensitive and specific method to identify *H. pylori* infection. The accuracy of the technique varies widely depending on the primers and target gene sequences, specimen preparation, bacterial density (Ashton-Key *et al.*, 1996; Wong *et al.*, 2001). There are no special requirements in the handling, transport and storage of the gastric biopsy samples and the results can be obtained in a short period of time. It can identify low numbers of bacteria present in specimens such as in saliva and faeces (Li *et al.*, 1996) or when the bacteria is no longer alive. PCR-based techniques have been developed to detect clarithromycin-resistant *H. pylori* strains from gastric biopsy samples by identification of mutations in the 23S rRNA gene (Chisholm *et al.*, 2001). However, it has a high risk of contamination resulting in false-positive results and it is technically demanding and not commonly available as a routine diagnostic tool.

## 1.6.2 Non-Invasive Tests

### 1.6.2.1 Serology

*H. pylori* infection induces cellular and humoral immune responses. There are various methods developed for the detection of antibodies to *H. pylori* in whole blood, serum, saliva and urine. Several antigens have been used such as whole cell sonicates and urease-enriched fractions. The measurement of specific antibodies in serum has been extensively used to determine colonisation status and can be used for the diagnosis of *H. pylori* infection as a screening procedure, or to follow the efficacy of eradication regimen. Specific IgM antibodies can be detected shortly after the infection is acquired, but IgA and IgG titres indicate chronic infection. Serological tests are commercially available, quick, easy to perform and inexpensive and have been recommended for the diagnosis of *H. pylori* infection in adults (Malfertheiner *et al.*, 1997). Among the various serological tests available, Enzyme-linked immunosorbent assay (ELISA) is the most widely used and has produced consistent and reliable results. Most assays detect specific IgG. Near-patient test kits provide a rapid diagnosis of *H. pylori* infection, which are based on latex agglutination or immunochromatography. They have low sensitivities compared with laboratory-based tests.

ELISA has low sensitivity for diagnosis of *H. pylori* infection in children, particularly the younger ones. The specific IgG antibody levels have been reported to be higher in older children (Raymond *et al.* 1996). Immunoblotting appears to be more sensitive and can detect low levels of antibodies that are not detected by ELISA (Nilsson *et al.*, 1997;

Rocha *et al.*, 2000). Furthermore, it allows the detection of antibodies to virulence factors such as CagA and VacA proteins. However, immunoblotting is expensive and time-consuming.

Antibodies to *H. pylori* persist in the blood for long periods of time. The reduction in the IgG antibody titre is not significant until the 6$^{th}$ month (Kosunen *et al.*, 1992). Therefore, the determination of the efficacy of therapy for *H. pylori* infection by serology requires several months for accurate detection of a significant fall in antibody titre. A preliminary study demonstrated that antibody to the heat shock protein (Hsp) hsp60 in serum declines rapidly after successful eradication and is useful for early assessment (4 weeks) of the eradication therapy (Yunoki *et al.*, 2000).

### 1.6.2.2 Urea Breath Test (UBT)

Urea breath test is currently the standard non-invasive test for the detection of *H. pylori* infection. It is a simple, safe and highly accurate method ideal to evaluate, in pre- and post-treatment conditions (Bazzoli *et al.*, 1997; Logan, 1998). The sensitivity and specificity are in the range of 97% and 95%. The test is based on the identification of urease activity of *H. pylori.* Urea labelled with either $^{13}C$ or $^{14}C$ is ingested by patients. In the presence of *H. pylori*, urease hydrolyses urea into ammonia and labelled bicarbonate, which is exhaled as labelled $CO_2$. The $^{13}C$ is detected by mass spectrophotometer while the $^{14}C$ is detected by scintillation counter. $^{13}C$-UBT is widely

used than $^{14}$C-UBT as $^{13}$C is non-radioactive isotope. Since the $^{13}$C-UBT is high cost, it is restricted to research centres. $^{14}$C-UBT has been reported to give unreliable result if the patients who had gastric surgery or have been treated with proton pump inhibitors or ranitidine (Chey *et al.*, 1997). To avoid false-negative results, a period of 7-14 days of PPI or $H_2$ antagonist treatment withdrawal is recommended before performing the UBT (Savarino *et al.*, 2000).

#### 1.6.2.3 Stool Antigen Test

The stool antigen detection is rapid, simple and easy for sample collection. The detection of *H. pylori* antigen in stool (HpSA) determined by ELISA has become commercially available. It is highly sensitive and specific. The accuracy of HpSA test is comparable to that of the urea breath test (Vaira *et al.*, 1999) and can be considered as an accurate and rapid method. It is easy for sample collection and can be used both in untreated patients and in the confirmation of *H. pylori* eradication after treatment. Since the antigen disappears quite rapidly, the measurement of HpSA is useful to assess the success of *H. pylori* eradication, which could be made as early as 2 weeks after treatment (Odaka *et al.*, 2002).

## 1.7 Treatment

The association between *H. pylori* and gastritis and peptic ulcer disease led to therapeutic attempts to eradicate this bacterium. *In vitro*, *H. pylori* is susceptible to various antimicrobial agents including penicillin, cephalosporins, macrolides,

tetracyclines, chloramphenicol, quinolones, rifampicin, carbapenems, nitroimidazoles, nitrofuran (Goodwin *et al.*, 1986; McNulty *et al.*, 1985; McNulty and Dent, 1988). Despite good *in vitro* activities, these antimicrobial agents, when they are used as single drugs, are ineffective *in vivo*. Treatment failure may be due to the lack of diffusion into the mucus, reduced activity in the acid environments (McNulty *et al.*, 1988) and acquired resistance to antibiotics such as quinolone and nitroimidazole (Glupczynski *et al.*, 1987; Goodwin *et al.*, 1988). Therefore, combination of antimicrobial agents is required for successful eradication. Triple therapy has been the best available and effective for *H. pylori* eradication.

Bismuth salts, such as bismuth subcitrate, are active against *H. pylori* but relapse of infection is common when used alone (Marshall *et al.*, 1987). Bismuth-based triple therapy, involving the administration of a bismuth salt plus metronidazole and either amoxycillin or tetracycline for 2 weeks, achieves eradication rates of up to 90% (Chiba *et al.*, 1992). However, this regimen is complicated, associated with adverse effects and partially overcome by metronidazole resistance.

The best current triple therapy involves proton pump inhibitor (PPI) or ranitidine bismuth citrate (RBC) with clarithromycin and either amoxicillin or metronidazole, which is considered as the first-line therapy (Malfertheiner *et al.*, 2002). Proton pump inhibitors (PPIs), such as omeprazole and lansoprazole, are active against *H. pylori in vitro* and inhibit acid secretion, leading to the increase of intragastric pH. Ranitidine bismuth citrate (RBC) is a new compound, which combines the antisecretory activity of

ranitidine with mucoprotective and *H. pylori* suppressive effects of bismuth. The second-line treatment, quadruple therapy, the addition of a PPI to bismuth triple therapy, has been recommended when the first-line treatment is unsuccessful. The regimen includes PPI, bismuth, tetracycline and metronidazole.

## 1.8 Macrolides

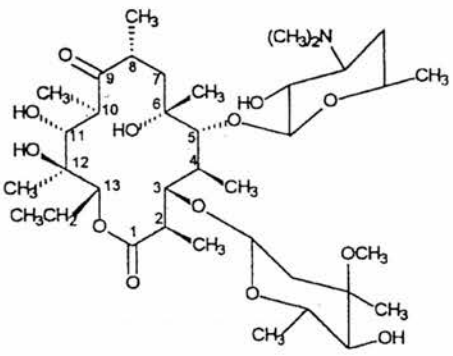
Macrolides have been widely used to treat various infections for over 40 years. Erythromycin, introduced in 1953, was the first macrolide antibiotic discovered and has been used for the treatment of respiratory tract and skin infections. It is also effective in the treatment of some intracellular pathogen infections caused by *Legionella, Mycoplasma* and *Chlamydia* species. Newer macrolides, such as clarithromycin, and azithromycin, have a broader spectrum of activities and better acid stability and phamacokinetic profiles. They have excellent activity against *Haemophilus influenzae*, *Mycobacterium avium* complex (MAC), *nontuberculous mycobacteria*, and *Chlamydia trachomatis*.

### 1.8.1 Structure-Activity Relationships

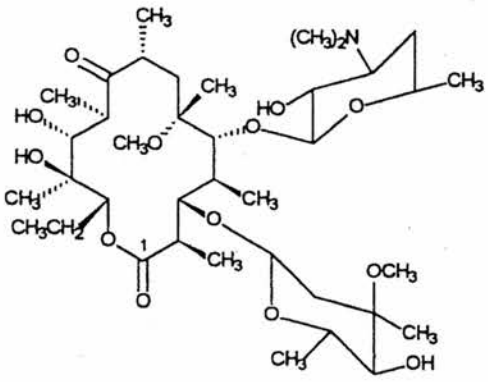
Macrolides, originally isolated from Streptomyces species, are composed of various amino sugars attached to a lactone ring of variable size (See Figure 1.1). Clinically useful macrolides consist of a 14-, 15-, and 16-membered lactone ring. Erythromycin is represented the first generation of the 14-membered ring macrolides. Modification of the

core erythromycin structure leads to differences in pharmacokinetic properties and in responses to bacterial resistant mechanisms (McFarland *et al.*, 1997). For instance, the semi-synthetic derivatives of erythromycin, clarithromycin, roxithromycin, and the 15-membered ring azithromycin, have better acid stability, broader spectrum of action and more potent activity against *H. influenzae* (Carbon, 1995). Ketolides, the latest generation of macrolides, are modified by replacing cladinose hydroxyl group with a ketone group. They exhibit an enhanced antibacterial activity and show a significant activity against some macrolide-resistant strains (Nilius *et al.*, 2001). Loss of bactericidal activity was, however, observed when the lactone ring of macrolides was cleaved. In addition, the absence of cladinose sugar in erythromycin or the tertiary amine groups of desosamine sugar in 14- and 15-membered ring macrolides (mycaminose sugar in 16-membered ring macrolides) leads to reduced antibacterial activities (Pestka and Lemahieu, 1974).

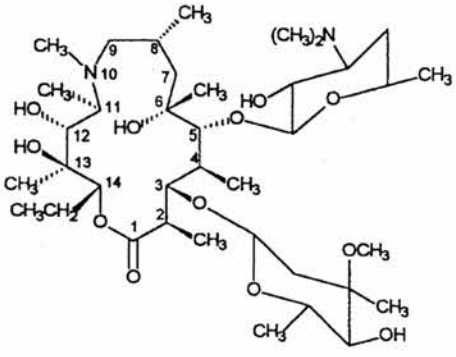
**Figure 1.1. Chemical structures of 14-membered ring macrolides, erythromycin, and clarithromycin, a 15-membered ring macrolide, azithromycin, and a ketolide, telithromycin.**



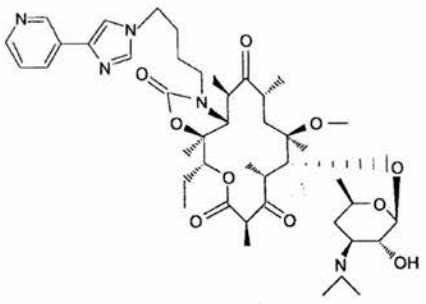
**Erythromycin**



**Clarithromycin**



**Azithromycin**



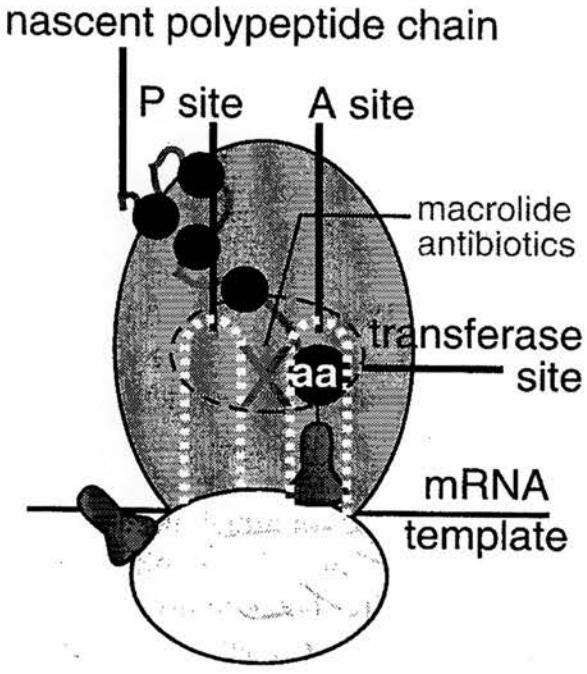
**Telithromycin**

### 1.8.2 Mechanism of Antibiotic Action

Macrolides are bacteriostatic agents that bind to the 50S ribosomal subunit and inhibit protein synthesis in the transpeptidation/translocation process by stimulating dissociation of the peptidyl-tRNA molecule from the ribosomes during elongation (Pruss *et al.*, 1999; Riesenfeld *et al.*, 1997) (See Figure 1.2). The precise mechanism of macrolide interaction with bacterial rRNA and the location of drug binding site have not yet been established. The peptidyltransferase loop of domain V and the hairpin 35 from domain II, two segments in the 23S rRNA, are assumed to be the major components of the macrolide binding site on the ribosome. The binding site of macrolides, chloramphenicol and clindamycin are overlapped (Cocito *et al.*, 1997; Vannuffel and Cocito, 1996).

Most of the 14-member-ring macrolides, including erythromycin and its derivatives, consist of three structural components: the lactone ring, the desosamine sugar, and the cladinose sugar. The hydrogen-bond interactions of macrolides with the peptidyl transferase region are mediated by the reactive groups of the desosamine sugar and the lactone ring (Gasc *et al.*, 1991; Steinmetz *et al.*, 1992). Schlunzen *et al.* showed that macrolides interacted with ribosome by seven hydrogen bonds and suggested that van der Waals forces, hydrophobic interactions and the geometry of the rRNA surrounding the macrolide molecules may involve in macrolide binding. The nucleotide A2058 appears to be crucial for drug binding since hydrogen bonds are formed between 2' OH of the desosamine sugar and N1 and N6 of A2058 (Schlunzen *et al.*, 2001). This can

**Figure 1.2. Inhibition of bacterial protein synthesis by macrolides.**

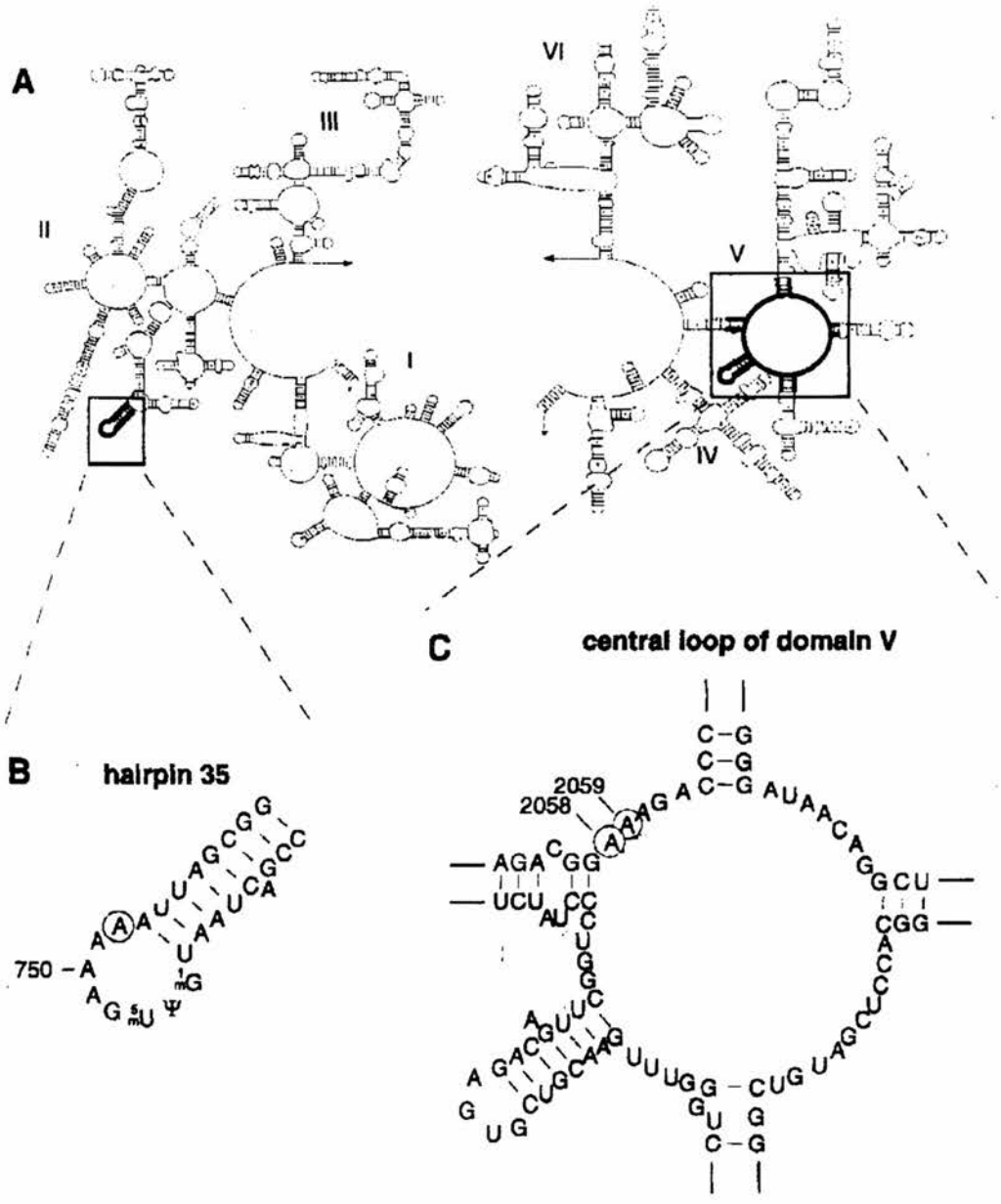


give explanation why the N6 dimethylation and rRNA mutations at A2058 confer macrolide resistance (Sigmund *et al.*, 1984; Weisblum, 1995).

Peptide bond synthesis occurs in the peptidyl transferase centre of the 50S subunit. Macrolides of erythromycin class do not inhibit peptidyl transferase activity. They bind to the 50S subunit at the entrance of the nascent peptide exit tunnel. It is thought that macrolides block the tunnel and cause dissociation of peptidyl-tRNA from the ribosome and subsequent cell death (Milligan and Unwin, 1986; Nissen *et al.*, 2000). Small peptides (up to 8 residues) are allowed to form before the nascent protein chain reaches the bound macrolides (Tenson *et al.*, 1996).

The interaction of macrolides with the 50S subunit is studied by RNA footprinting. It has been demonstrated that macrolides interact with hairpin 35 in domain II and the peptidyl transferase loop in the domain V of the 23S rRNA (Hansen *et al.*, 1999; Xiong *et al.*, 1999) (See Figure 1.3). Several studies demonstrated that macrolides protected A2058 and A2059 of the 23S rRNA from chemical modification (Beauclerk and Cundliffe, 1987; Hansen *et al.*, 1999; Moazed and Noller, 1987; Xiong *et al.*, 1999). It has also been revealed that macrolides interact with the helix 35 in domain II of the 23S rRNA , suggesting that peptidyl transferase cavity and hairpin 35 involve in drug binding and may be folded closed together in the 23S rRNA tertiary structure (Hansen *et al.*, 1999; Xiong *et al.*, 1999). However, by using the high-resolution X-ray structure of the 50S rRNA, Schlunzen *et al.* demonstrated that macrolides bound to the peptidyl

**Figure 1.3. Secondary-structure models of 23S rRNA (A) the hairpin 35 (B) and the peptidyl transferase centre in domain V of 23S rRNA (C) are shown in enlarged (Adapted from Xiong *et al.* 1999).**



transferase cavity of the 23S rRNA at the entrance of the tunnel and no direct interaction was found between macrolides and helix 35. It was indicated that the footprinting effect at A752, in Domain II, might be of an allosteric effect (Schlunzen *et al.*, 2001). Mutations in two ribosomal proteins, L4 and L22 confer resistance (Arevalo *et al.*, 1988; Chittum and Champney, 1994), 23S rRNA, (Tait-Kamradt *et al.*, 2000; Wittmann *et al.*, 1973). It is still unclear, however, whether these ribosomal proteins directly play a role in the drug binding or the mutations perturb the conformation in the drug-binding site (Gabashvili *et al.*, 2001; Gregory and Dahlberg, 1999). In spite of these advances in the understanding of the mode of action of macrolides, the exact process remains unknown.

## 1.8.3 Mechanism of Macrolide Resistance

Erythromycin resistance was described in bacterial pathogens a few years after erythromycin was introduced in the 1950s. Resistance to macrolides can occur by several mechanisms, including alterations in the drug target site, drug inactivation and active efflux.

### 1.8.3.1 Target Site Alterations

#### 1.8.3.1.1 Mutations in Ribosomal Proteins

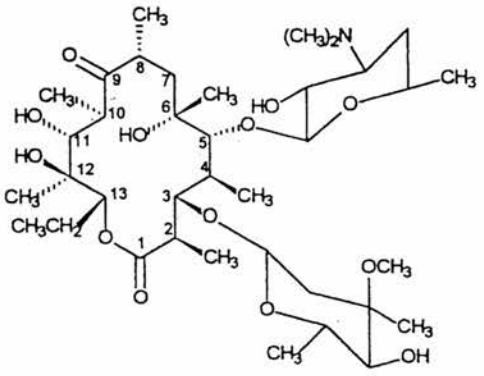
Mutations in ribosomal proteins, L4 and L22, have been linked to erythromycin resistance (Wittmann *et al.*, 1973). However, evidence from structural study by Schlunzen demonstrated that there was no direct interaction between the drugs and

ribosomal proteins (Schlunzen *et al.*, 2001). Mutations in these two ribosomal proteins conferring resistance is, presumably mediated by perturbing the conformation of the 23S rRNA (Gregory and Dahlberg, 1999).

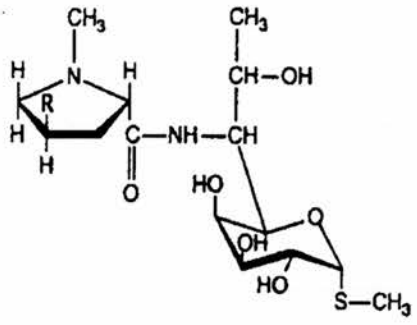
#### 1.8.3.1.2 Posttranscriptional Methylation of A2058 in the 23S rRNA

Different bacterial species can produce an enzyme, encoded by erythromycin-resistant methylase (*erm*) genes, which methylate the N-6 position of adenosine 2058 (*E. coli* numbering) of the 23S rRNA to form either mono- or dimethyladenine. This results in conformational changes in the P site of the rRNA and prevents the macrolide binding. The modification by methylase leads to cross-resistance in the macrolides lincosamides and streptogramin B antibiotics. This phenomenon was termed the macrolide-lincosamide-streptogramin B ($MLS_B$) antibiotic resistance phenotype. Macrolide, lincosamide and streptogramin antibiotic are chemically distinct but have similar mode of action (See Figure 1.4). These antibiotics act as protein synthesis inhibitors by blocking the peptidyl transferase region of the 50S subunit of the bacterial ribosome. $MLS_B$ cross-resistance phenotype is probably due to the overlapping binding sites of the $MLS_B$ antibiotics. The $MLS_B$ resistance has been identified in *Staphylococcus* spp., *Streptococcus* spp., *Enterococcus* spp., *Corynebacterium diptheriae*, *Bacteroides* spp.,

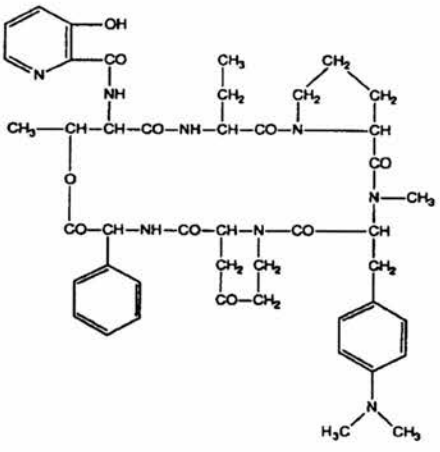
**Figure 1.4. The MLS superfamily antibiotics. Chemical structures of erythromycin, lincomycin, a lincosamide, and streptogramin B.**



**Erythromycin**



**Lincomycin**



**Streptogramin B**

*Clostridium* spp., *Bacillus* spp., *Lactobacillus* spp., *Mycoplasma pneumoniae*, *Campylobacter* spp., and recently, *Propionibacterium* spp., and members of family *Enterobacteriaceae* (Leclercq and Courvalin, 1991).

Some *erm* genes are inducible. The 15- and particularly the 14-membered ring macrolides are strong inducers. It was demonstrated that the basal levels of ribosomal methylation varied from strain to strain in *erm*-bearing *S. pneumoniae* clinical isolates (Rosato *et al.*, 1998). This is due to the different ability of the macrolides and structurally related antibiotics to induce rRNA methylation. Nine *erm* genes from various clinically important bacterial species, belonging to four hybridisation classes, have been recognised (Table 1.2) (Pechere, 2001).

**Table 1.2. Erythromycin-resistant methylase (*erm*) genes in clinically important bacteria**

| Gene | Bacterial species |
|---|---|
| *ermA* | *S. aureus*, coagulase-negative staphylococci |
| *ermP* | *C. perfringens, C. difficile, E. faecalis* |
| *ermZ* | *C. difficile, E. faecalis* |
| *ermBC* | *L. reuteri, E. coli* |
| *ermAM* | *S. sanguis, S. pneumoniae* |
| | *S. agalactiae, S. pyogenes* |
| *ermB* | *S. aureus, B. subtilis* |
| *ermC* | *S. aureus,* coagulase-negative staphylococci |
| *ermM* | *S. epidermidis* |
| *ermF* | *B. fragilis, B. avatus* |

#### 1.8.3.1.3 Mutations in the 23S rRNA

The 23S rRNA mutations confer macrolide resistance by altering the structure of the drug binding site and reducing the interaction between macrolides and ribosomes. Mutations conferring macrolide resistance are listed in Table 1.3 (Vester and Douthwaite, 2001).

The 23S rRNA mutation at A2058, the same site for methylation, leads to $MLS_B$ resistance. It was first identified in *Mycobacterium intracellulare* (Meier *et al.*, 1994). Mutational alteration in 23s rRNA has been reported in *Mycobacterium avium* (Sander *et al.*, 1996) and *H. pylori* (Versalovic *et al.*, 1996) , and other organisms containing only one or two 23S rRNA genes (Tait-Kamradt *et al.*, 2000). It is unusual to find this type of mutation in clinically resistant isolates carrying multiple copies (i.e. more than four) of the 23S rRNA gene such as staphylococci and streptococci (Davies *et al.*, 2000; Gasc *et al.*, 1991). Mutations at A2058, or at A2059 confer the highest levels of resistance. Mutations at positions 2057, 2452, and 2611 have lower-level drug resistance. Despite being slightly outside the central point of macrolide interaction, these positions are close by in the secondary structure.

Macrolide resistance was observed during antibiotic therapy for eradication of *H. pylori*. Mutations at positions A2058 or A2059 in the 23S rRNA were identified in clarithromycin-resistant isolates (Versalovic *et al.*, 1996). Taylor *et al.* reassigned the

positions 2058 and 2059 as positions 2142 and 2143 based on the precise location in the 23 s rRNA of *H. pylori* (Taylor *et al.*, 1992). Other macrolide-resistant mechanism including *erm* genes or macrolide efflux systems has not yet been reported in *H. pylori* (Debets-Ossenkopp *et al.*, 1996; Hulten *et al.*, 1997). Mutations at A2058 and A2059 conferring erythromycin-resistance were reported in *Mycoplasma pneumoniae* isolates (Lucier *et al.*, 1995). These mutations were also observed in propionibacteria clinical isolates while a G2057 mutation was found in some isolates (Ross *et al.*, 1997).

In domain II, mutation at position 754 in hairpin 35 confers low-level macrolide resistance in an *E. coli* laboratory strain, suggesting the proximity of this hairpin and the peptidyl transferase region in the 23S rRNA tertiary structure (Xiong *et al.*, 1999). Douthwaite demonstrated that deletion of selected nucleotides in domain II in laboratory mutants confers erythromycin resistance and suggested that rRNAs in domains II and V interact through a ribosomal protein (Douthwaite, 1992).

**Table 1.3. 23S rRNA mutations reported to confer macrolide resistance**

| *E. coli* 23S rRNA position | Organism |
|---|---|
| 754 | *Escherichia coli* |
| 2057 | *Escherichia coli*<br>*Propionibacteria* |
| 2058 | *Escherichia coli*<br>*Helicobacter pylori*<br>*Mycobacterium abscessus*<br>*Mycobacterium avium*<br>*Mycobacterium chelonae*<br>*Mycobacterium intracellulare*<br>*Mycobacterium kansasii*<br>*Mycobacterium smegmatis*<br>*Mycoplasma pneumoniae*<br>*Propionibacteria*<br>*Streptococcus pneumoniae*<br>*Streptomyces ambofaciens*<br>*Treponema pallidum* |
| 2059 | *Helicobacter pylori*<br>*Mycobacterium abscessus*<br>*Mycobacterium chelonae*<br>*Mycobacterium intracellulare*<br>*Mycobacterium avium*<br>*Mycobacterium smegmatis*<br>*Mycoplasma pneumoniae*<br>*Propionibacteria* |
| 2452 | *Sulfolobus acidocaldarius* |
| 2611 | *Escherichia coli*<br>*Streptococcus pneumoniae* |

### 1.8.3.2 Antibiotic Modification

Macrolide-modifying enzymes including erythromycin esterase (Barthelemy *et al.*, 1984) and macrolide 2'-phosphotransferase (O'Hara *et al.*, 1989) are produced by the strains inactivating the lactone ring of the 14-membered macrolides. Two types of esterases, I and II, encoded by *ereA* (Ounissi and Courvalin, 1985) and *ereB* (Arthur *et al.*, 1986) has been identified. Esterase genes have been reported in *E. coli*, *Klebseilla*, *Citrobacter*, *Proteus* and *Enterobacter* spp. A clinical isolate of *S. aureus* was found to inactivate 14- and 16-membered-ring macrolides (Wondrack *et al.*, 1996). The products of inactivation were identical to products produced by *E. coli*, encoded for either EreA or EreB esterase.

### 1.8.3.3 Active Efflux

Efflux is one of the resistance mechanisms for macrolides mediated by actively transporting the antibiotic out of the cell, thus keeping the intracellular concentration low and ribosomes free from antibiotic. Different efflux systems conferring macrolide resistance have been described. The *msr*A efflux pump, encoded by plasmid-borne *msr*(A) genes, is responsible for macrolide and streptogramin B resistance in staphylococci. It is a member of the ATP-binding-cassette (ABC) transporter superfamily (Ross *et al.*, 1990). The *mef* genes have been found in a variety of Gram-positive bacteria, including corynebacteria, enterococci, micrococci, and streptococci (Kataja *et al.*, 1998; Luna *et al.*, 1999; Shortridge *et al.*, 1996). Two *mef* genes, including *mef* (A)(Clancy *et al.*, 1996) and *mef*(E) (Tait-Kamradt *et al.*, 1997), have

been described. The Mef protein belongs to the major facilitator superfamily (MFS), which is fuelled by the proton motive force. This efflux appears to be chromosome-mediated and affects only macrolides but not lincosamide and streptogramins. The *mef*(A) gene was described in *Streptococcus pyogenes* (Clancy *et al.*, 1996) while the *mef*(E) gene was reported in *S. pneumoniae* (Tait-Kamradt *et al.*, 1997) .

### 1.8.4 Mechanism of Macrolide Resistance in the Clinic

In the USA, over two-thirds of the macrolide-resistant *S. pneumoniae* strains harboured the efflux mechanism (Gay *et al.*, 2000; Shortridge *et al.*, 1999). The other third of the resistant isolates contained the rRNA modification mechanism. A study conducted in Canada (Johnston *et al.*, 1998) demonstrated that the *mef*E gene was detected more frequently than *erm* genes in *S. pneumoniae*. In most *S. pneumoniae* strains isolated in Belgium and Italy, macrolide resistance is caused by the presence of the *erm* genes (Descheemaeker *et al.*, 2000; Oster *et al.*, 1999). In Japan, it was described that approximately 60% of erythromycin-resistant *S. pneumoniae* strains harboured the *erm*B gene, whereas 40% carried the *mef*E gene (Nishijima *et al.*, 1999). In Western Europe, efflux mechanism was observed in the majority of the erythromycin-resistant *S. pyogenes* isolates (Alos *et al.*, 2000; Descheemaeker *et al.*, 2000; Giovanetti *et al.*, 1999).

Mutations at A2058 (or neighboring nucleotides) in the 23S rRNA have been demonstrated in macrolide-resistant clinical strains of *Mycobacterium avium* (Sander *et al.*, 1996) and *H. pylori* (Versalovic *et al.*, 1996), and other organisms harbouring only one or two 23S rRNA genes (Tait-Kamradt *et al.*, 2000). Resistance in bacteria with multiple 23S rRNA operons, such as *Enterococcus*, *Streptococcus*, and *Staphylococcus* species, is generally conferred by Erm methylation of A2058 or by efflux (Kataja *et al.*, 1999; Ross *et al.*, 1990). Antibiotic resistance in clinical strains can be linked to alterations of specific nucleotides of the 23S rRNA within the peptidyl transferase centre (Vester and Douthwaite, 2001). Mutations of A2058 (*E. coli* numeration) and several neighboring positions in the central loop of domain V confer resistance to macrolides (Sigmund and Morgan, 1982).

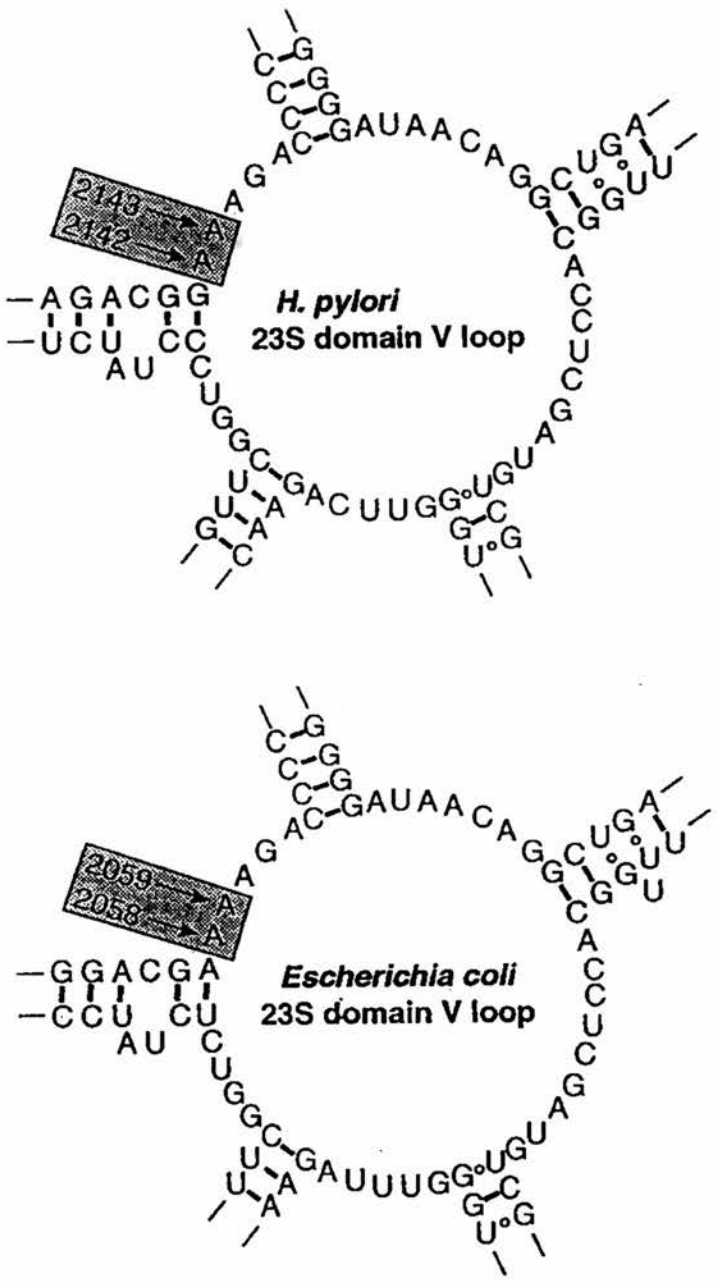
### 1.8.5 Clarithromycin Resistance in *H. pylori*

Macrolides are essential component for *H. pylori* eradication regimen. Among the macrolides available, clarithromycin is currently widely used. It has a low MIC against *H. pylori* and good diffusion in the gastric mucosa. The antimicrobial activity is less affected by a decreased in pH than that of the other compounds in this group. The antimicrobial effect of clarithromycin, in common with that of established macrolides, has been attributed to binding to the 50S ribosomal subunit within the bacterial cell, thus inhibiting translocation of aminoacyl transfer-RNA and consequent protein synthesis. Clarithromycin resistance in *H. pylori* is associated with mutations in the peptidyl transferase region of the 23S ribosomal RNA genes (Versalovic *et al.*, 1996). Mutations

in the 23S rRNA decrease the affinity of clarithromycin for the ribosome and thus interfere with antimicrobial activity (Occhialini *et al.*, 1997).

Mutations conferring macrolide resistance were first described in two genes positions 2142 or 2143, formerly named 2058 and 2059, respectively, which are the cognates in *E. coli,* then 2143 and 2144 before to be revised as it is now (Taylor *et al.*, 1997). The point mutation can be a transition (A to G) or a transversion (A to C). Versalovic *et al.* found that mutation from A to G at position 2142 is the most frequent (Versalovic *et al.*, 1997), whist Domingo *et al.* demonstrated that the A to G mutation at postion 2143 is the most frequent mutation (Domingo *et al.*, 1998). A number of studies reported mutations conferring macrolide resistance in *H. pylori* and at least five distinct point mutations (G2115 to A, G2141 to A, A2142 to G, A2142 to C, and A2143 to G) have been reported (Debets-Ossenkopp *et al.*, 1998; Garcia-Arata *et al.*, 1999; Hulten *et al.*, 1997; Occhialini *et al.*, 1997; Stone *et al.*, 1996; Versalovic *et al.*, 1997; Wang and Taylor, 1998).

**Figure 1.5 Comparison of the domain V loops of the 23 S rRNA from *H. pylori* and *E. coli* (Figure taken from Taylor *et al.* 1997).**



## 1.9 Metronidazole

Metronidazole, 1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole) is a synthetic antibacterial and antiprotozoal agent that belongs to the nitroimidazole class (See Figure 1.6). It was first introduced for the treatment of gynaecological infections caused by *Trichomonas vaginalis* in 1959. Metronidazole has been found to be active against anaerobic and some microaerophilic bacteria such as *H. pylori*. It is now widely used for treatment of infections with anaerobic microorganisms (Edwards, 1993; Ingham *et al.*, 1980; Muller, 1983).

### 1.9.1 Mechanism of Action

Metronidazole is amebicidal, bactericidal and trichomonicidal. The drug itself is not toxic. It has to enter the cell and requires reductive activation of the nitro group of the imidazole ring to form a hydroxylamine derivative. The reduced product causes DNA degradation and strand breakage (Edwards, 1993; Lindmark and Muller, 1976). Metronidazole needs an extremely low redox potential to be converted itself into an active form. Therefore, the selective toxicity of metronidazole for anaerobic bacteria and protozoa is due to the low redox potential of the components of their electron transport proteins, such as ferredoxin and flavodoxin, which have sufficiently low redox potential to reduce metronidazole to its toxic nitroradical. In anaerobic organism, electrons derived from oxidative decarboxylation of pyruvate, catalysed by POR (pyruvate oxidoreductase) are transferred to ferredoxin or flavodoxin. By having very low redox potential, metronidazole accepts electron from the reduced electron carrier proteins and is then activated (Edwards, 1993; Edwards *et al.*, 1973) (See Figure 1.7).

**Figure 1.6. The chemical structure of metronidazole**

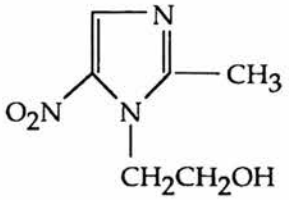
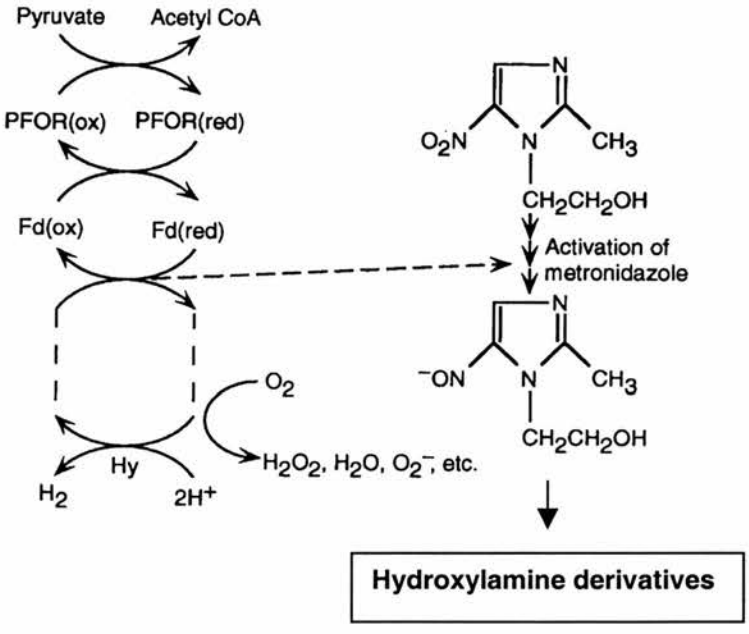


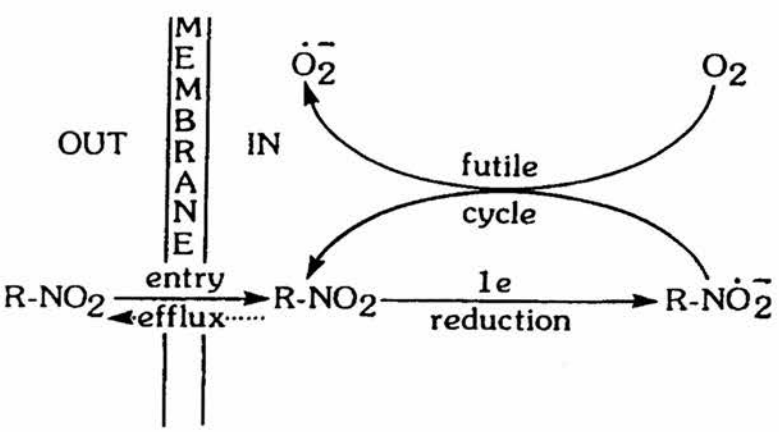

**Figure 1.7. Scheme of the interaction of metronidazole with electron transport components (Figure adapted from Quon *et al.* 1992)**




Anaerobic organisms develop metronidazole resistance by reducing or abolishing activity of elements of this series of electron transport reactions, particularly POR and ferrodoxin, with appropriate compensatory modifications of the normal fermentative pathway (Edwards, 1993; Moreno *et al.*, 1983; Narikawa, 1986; Quon *et al.*, 1992).

An alternative mechanism of action of metronidazole in anaerobic bacteria exposed to aerobic conditions has been proposed. The presence of molecular oxygen may oxidise the nitro anion radicals generated from the reduction of metronidazole, resulting in a one-electron transfer step back to the parent compound (Moreno *et al.*, 1983) (Figure 1.8). Regeneration of the parent compound would then lead to further cycles of reduction and oxidation. Although this process, term 'futile cycling' results in detoxification of the drug, it also generates superoxide radical anions that may be toxic to the microorganism. Under normal circumstance, superoxide dismutase converts superoxide anions to hydrogen peroxide, which is further reduced to water by the action of catalase. In the presence of transition metals, such as iron or copper, superoxide ions and hydrogen peroxide may react to generate the highly reactive, DNA damaging hydroxyl free radical (Edwards, 1993). Increased expression of superoxide dismutase has been reported in metronidazole-resistant amoebae and appears to contribute to the resistant phenotype, rather than the result of a general stress response.

**Figure 1.8. Futile cycling (Figure taken from Edwards, 1993)**



### 1.9.2 Resistance to Metronidazole in *H. pylori*

Since metronidazole is used for the treatment of many infections caused by anaerobic bacteria and protozoa, an increase of metronidazole-resistant *H. pylori* strains has been generated. The mechanism of resistance is still unclear. Several mechanisms have been proposed including reduced uptake and/or increased efflux of metronidazole (Lacey *et al.*, 1993), inactivation of metronidazole through 'futile cycling' (Cederbrant *et al.*, 1992), increased repair of radical-induced damage (Chang *et al.*, 1997; Jorgensen *et al.*, 1998; Smith and Edwards, 1995) and mutation in metronidazole-reducing enzymes (Goodwin *et al.*, 1998; Hoffman *et al.*, 1996; Kaihovaara *et al.*, 1998).

Since *H. pylori* live in microaerobic environment, it was proposed that the reactive oxygen species generated by 'futile cycling' were responsible for cell death (Edwards, 1993; Lacey *et al.*, 1993). In the presence of oxygen, reduced metronidazole can convert back to its parent compound by 'futile cycling' process, generating superoxide anions instead of hydroxylamine. The increased synthesis of superoxide dismutase and catalase has been hypothesised to explain metronidazole activity in *H. pylori* (Cederbrant *et al.*, 1992). However, the presence of this process has not yet been successfully demonstrated (Jorgensen *et al.*, 1998).

Smith and Edwards (Smith and Edwards, 1995) demonstrated that superoxide dismutase and catalase were not induced by metronidazole and there was no correlation between

enzyme levels and resistance patterns. Jorgensen found that in the presence or absence of catalase activity has no effect on the susceptibility of strains to this antibiotic (Jorgensen *et al.*, 1998). Little evidence has been demonstrated that futile cycling contributes to the mode of action of metronidazole. Therefore, futile cycling does not occur in *H. pylori*.

Microaerophilic bacteria oxidise pyruvate via pyruvate:ferredoxin/flavodoxin oxidoreductase (POR), metabolic feature found in anaerobic bacteria (Hughes *et al*, 1995). Both anaerobic and microaerophilic bacteria are susceptible to 5-nitroimidazole drugs (Hof *et al.*, 1984; Lockerby *et al.*, 1985; Smith and Edwards, 1995). These drugs, when reduced by low-potential ferredoxins or flavodoxins, form hydroxylamine derivatives, which damage DNA (Kedderis *et al.*, 1988; Krieg and Hoffman, 1986; Lindmark and Muller, 1976; Muller, 1983; Smith and Edwards, 1995).

The loss of resistance in metronidazole-resistant *H. pylori* strains on exposure to anaerobic environment was demonstrated (Smith and Edwards, 1995). It is suggested that the mechanism of resistance is probably mediated through the activation of anaerobic metabolic pathways, which function less or not at all under microaerophilic conditions, causing the reduction of metronidazole, thus accounting for the increased sensitivity to drug (Smith and Edwards, 1995).

Furthermore, Smith and Edwards studied the ability of metronidazole susceptible and resistant strains to scavenge oxygen from intracellular environment and found that metronidazole resistant *H. pylori* strains had reduced activity of NADH oxidase, an oxygen scavenger (Smith and Edwards, 1997). Metronidazole resistance may be mediated through an inability of metronidazole-resistant strains to remove $O_2$ from the site of metronidazole reduction, thus preventing metronidazole activation. Jorgensen demonstrated that metronidazole-resistant *H. pylori* reduced metronidazole more slowly than their sensitive counterparts. These results strongly suggest that *H. pylori* is capable of reducing metronidazole to its active form at the relative high redox potentials encountered in microaerobic conditions and that this process is reduced or inactive in resistant strains. Hoffman *et al.* demonstrated an association between metronidazole resistance and repression of the pyruvate oxidoreductase and α–ketoglutarate oxidoreductase activities in response to low levels of metronidazole in *H. pylori* (Hoffman *et al.*, 1996).

*H. pylori* contains a NADPH nitroreductase, encoded by *rdxA* gene, which reduce metronidazole through sequential two-electron reductions to generate toxic metabolites that cannot be transformed to its parent compound under aerobic or microaerophilic conditions by the 'futile cycling' process. Goodwin *et al.* demonstrated that that the loss of oxygen-insensitive NADPH nitroreductase activity was responsible for the mechanism of metronidazole resistance in *H. pylori*, and mutational inactivation of the *rdxA* gene conferred resistance (Goodwin *et al.*, 1998).

Debets-Ossenkopp also confirmed that *rdxA* gene was disrupted in metronidazole-resistant *H. pylori* strain NCTC11637 and no mutation was detected in any other genes suggested to be linked to the mechanism of resistance (Debets-Ossenkopp *et al.*, 1999). Jenk *et al.* studied the role of *rdxA* in a mouse model and demonstrated that 25 out of 27 metronidazole-resistant strains isolated from mice had mutations in the *rdxA* gene (Jenks *et al.*, 1999). Tankovic *et al.* reported mutation in the *rdxA* gene in 12 of 13 metronidazole-resistant clinical isolates (Tankovic *et al.*, 2000).

The *rdxA* gene of some metronidazole-resistant strains appears to be unchanged (Jenks *et al.*, 1999; Tankovic *et al.*, 2000). This suggests that other mechanisms of resistance may exist in *H. pylori.* The contribution of other mechanisms responsible for resistance is still unknown. Mutations in the genes regulating the expression of the *rdxA* gene or in the promoter sequence of the *rdxA* gene have been postulated (Van Der Wouden *et al.*, 2000). Metronidazole efflux or reduced uptake, deficiency of other enzymes involved in the reduction of metronidazole to its active form, target modification or increased DNA repair may play a role in the mechanism of resistance.

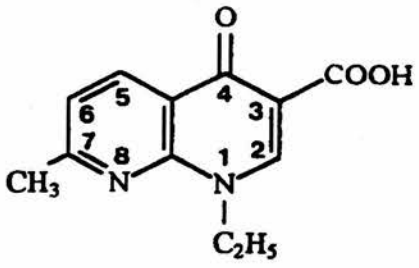
More recently, inactivation of other reductase-encoding genes, including *frxA*, encoding NADPH flavinoxidoreductase, and *fdxB*, encoding ferredoxin-like protein, have been observed to be associated with metronidazole resistance (Jeong *et al.*, 2000; Kwon *et al.*, 2000).

## 1.10 Fluoroquinolones

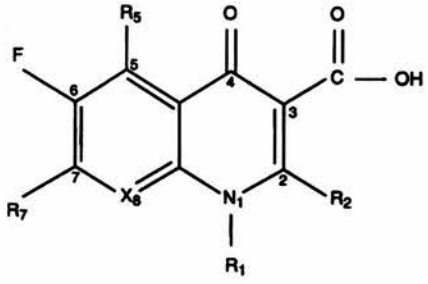
Fluoroquinolones are a series of synthetic antibacterial compounds used in the treatment of a wide range of bacterial infections. Nalidixic acid, the first clinically useful quinolone, was discovered as a by-product of the purification process of the anti–malarial drug chloroquine (Lesher *et al.*, 1962). It had good activity against certain Gram-negative bacteria, particularly the *Enterobacteriaceae* but showed only poor activity against Gram-positive bacteria. As a result of its limited spectrum of activity and poor serum and tissue concentrations, the clinical applications of nalidixic acid was restricted. It was not until almost 20 years later that the substitution of a fluorine atom at position C-6 of the quinolone core structure was synthesised, leading to the construction of fluoroquinolones (Koga *et al.*, 1980). Since then hundreds of quinolones have been synthesised and this significant breakthrough generated a new spectrum of compounds with improved absorption and enhanced antibacterial potency. Figure 1.9 shows the structures of nalidixic acid and fluoroquinolone core.

Structures of quinolones (Figure 1.10) have developed along two major pathways: the naphthyridones, which contain original naphthyridine core of nalidixic and the fluoroquinolones, which have a carbon atom substituted for nitrogen at the 8-position of the naphthyridine core (Domagala, 1994). All of them contain a fluorine substitution at position 6. The C-7 position is the most adaptable site for chemical change. The characteristics of quinolone generations are shown in Table 1.4 (Ball, 2000).

**Figure 1.9. Nalidixic acid and fluoroquinolone core**

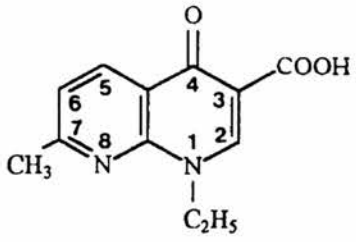



**Nalidixic acid**

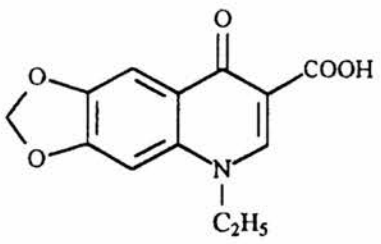



**Fluoroquinolone core**

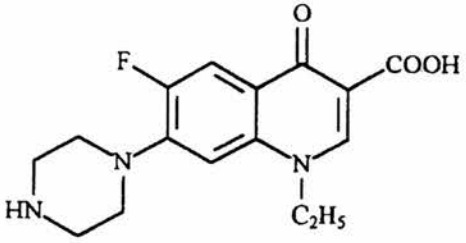
**Figure 1.10. Structures of nalidixic acid and other representative quinolones**



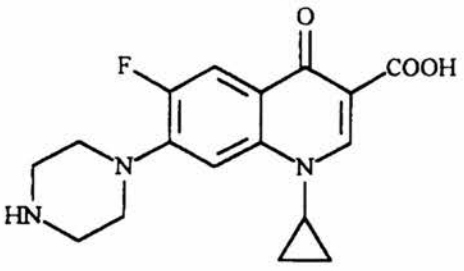

Nalidixic acid



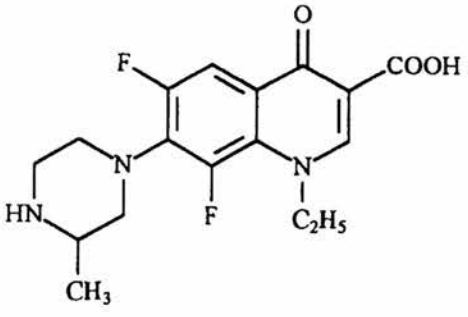

Oxolinic acid



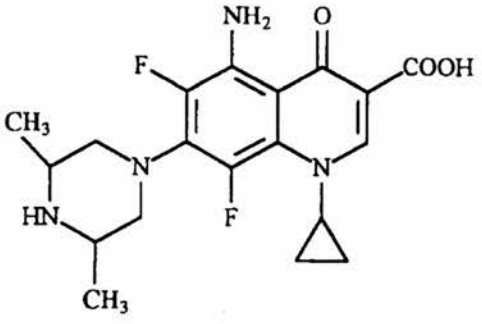

Norfloxacin




Ciprofloxacin




Lomefloxacin




Sparfloxacin

**Table 1.4. Characteristics of quinolone generations. (Adapted from Ball *et al.*, 2000)**

| | Core structure | | Characteristics |
|---|---|---|---|
| **Generation** | **Fluoroquinolone** | **Naphthyridone** | |
| I | flumequine | nalidixic acid | predominantly used for the treatment of urinary tract infections |
| IIa | ciprofloxacin, ofloxacin (levofloxacin) | enoxacin | enhanced activity mainly against Gram-negative pathogens. |
| IIb | grepafloxacin, sparfloxacin | tosufloxacin | balanced broad spectrum of activity, increased potency against pneumococci. |
| IIIa | moxifloxacin gatifloxacin sitafloxacin clinafloxacin | trovafloxacin | enhanced activity against Gram-positive pathogens |
| IIIb | not yet developed | gemifloxacin | markedly enhanced activity against Gram-positive pathogens |

The second-generation fluoroquinolones, such as ciprofloxacin, have predominantly activity against Gram-negative bacteria but less effective against Gram-positive bacteria including *S. pneumoniae* and methicillin-resistant *S. aureus*. The development of enhanced Gram-positive activity has been shown in the generation IIb compounds such as grepafloxacin and sparfloxacin. The third-generation fluoroquinolones, such as gatifloxacin (Perry *et al.*, 1999) and moxifloxacin (Dalhoff *et al.*, 1996), have enhanced activity against Gram-positive pathogens and are very active against *S. pneumoniae*. They are highly effective in lower respiratory tract infection treatment. Gemifloxacin has markedly better activity against Gram-positive bacteria, particularly *S. pneumoniae*, than any other quinolones (Cormican and Jones, 1997).

Fluoroquinolones are potent broad-spectrum antimicrobial drugs that target two essential enzymes in the bacterial cell, type II topoisomerases, DNA gyrase and topoisomerase IV (Levine *et al.*, 1998). They have bactericidal activities as DNA gyrase and topoisomerase IV are required for cell growth and division.

### 1.10.1 Bacterial Topoisomerases

Topoisomerases fall into two categories: Type I and Type II. All topoisomerases have the abilities to change the topology of DNA through breakage and reunion of DNA strands. They have, however, different activities. Type I topoisomerases comprise topoisomerase I and III which are able to relax and decatenate interlocked DNA circles by catalysing single stranded DNA cleavage and strand passage in the absence of ATP. Type II topoisomerases include DNA gyrase and topoisomerase IV which are multi-subunit enzymes and ATP dependent. Unlike type I topoisomerases, DNA gyrase and topoisomerase IV alter DNA topology by introducing a double-strand breakage with the passage of another helix through the transient break and religating the broken ends (Drlica and Zhao, 1997). This process is essential in maintaining the appropriate state of DNA supercoiling and decatenation of DNA during cell replication. DNA gyrase and topoisomerase IV are targets of fluoroquinolones.

### 1.10.2 DNA Gyrase

DNA gyrase consists of two-subunits (an $A_2B_2$ tetramer), encoded by the *gyrA* and *gyrB* genes (Swanberg and Wang, 1987). DNA Gyrase introduces negative supercoils into relaxed DNA, and thus relieves topological stress arising from the translocation of transcription and replication complexes along DNA (Reece and Maxwell, 1991). It can also unlink the knots and catenanes generated by recombination. Gyrase, however, does not play a major role in unlinking catenanes formed during replication and topoisomerase IV is principally responsible for this function.

In *E. coli*, GyrA, a 97 kDa fragment, mediates strand breakage and reunion. The active sites, tyrosine 122, bind to the DNA phosphate backbone and perform cleavage and religation (Horowitz and Wang, 1987). GyrB, a 90-kDa fragment in *E. coli*, functions by supporting DNA relaxation and is responsible for ATP binding and hydrolysis (Reece and Maxwell, 1991). The N-terminal region of GyrA interacts with GyrB, whereas the C-terminal domain is responsible for DNA supercoiling. Similarly, the N-terminus of GyrB contains the ATP-binding domain, and the C-terminus contains the GyrA interaction region.

### 1.10.3 Topoisomerase IV

Topoisomerase IV, a $C_2E_2$ complexes, consists of *parC* and *parE* genes. ParC and ParE are homologous to the A and B subunits of DNA gyrase, respectively. Topoisomerase IV, a decatenating enzyme, resolves interlinked daughter chromosomes following DNA replication. It can relax negatively supercoiled DNA at a much slower rate than decatenation (Hiasa and Marians, 1996). ParC is 36% identical to GyrA, while Par E is 40% identical to GyrB (Kato *et al.*, 1990). The active sites, tyrosine 120 in ParC, bind to the DNA phosphate backbone and perform cleavage and religation, as GyrA activity. ParE provides similar activities as GyrB, responsible for ATP binding and hydrolysis.

DNA gyrase and topoisomerase IV use a double-strand-passage mode of action (Roca, 1995) but have a difference in DNA wrapping. Gyrase wraps DNA around itself whereas topoisomerase IV does not (Peng and Marians, 1995). Topoisomerase IV favors

intermolecular strand passage reactions such as decatenation while gyrase favor intramolecular strand passage reaction such as supercoiling.

### 1.10.4 Drug-Target Interaction

Since mutations in the *gyrA* gene confer quinolone resistance, it was believed that quinolone bound directly to GyrA (Gellert *et al.*, 1977). The occurrence of *gyrB* mutations suggested that the quinolone binding site may include both subunits (Yamagishi *et al.*, 1986). Shen *et al.* proposed a cooperative drug-binding model in which a quinolone binds to bases of single-stranded DNA and self-associates in a pocket created by gyrase (Shen *et al.*, 1989) (Figure 1.11). However, this model cannot explain why mutation changes in the gyrase genes can cause quinolone resistance. The quinolones are believed to interact with the bases through hydrogen bonds between the 3-carboxy and 4-oxo groups. At least 4 molecules are thought to bind per binding site and ring stacking and hydrophobic interactions contribute to their cooperativity. Shen *et al.* postulated that gyrase interacts with the C-7 position of the drug (Shen *et al.*, 1989), however, Maxwell later pointed out that the C7 group is variable in the quinolones (Maxwell, 1992). Yoshida et al proposed a quinolone pocket model in which quinolones exerts their action through their interaction in a pocket of the gyrase-DNA complex that appears during DNA cleavage-reunion reactions and the quinolone binding affinities for the complex are mediated by both GyrA and GyrB (Yoshida *et al.*, 1993).



Palumbo *et al.* proposed an alternative model, involving drug binding to phosphate groups in DNA via a $Mg^{2+}$ bridge, stacking interactions between the quinolone and a DNA base in a single stranded region, stabilised with metal ion (Palumbo *et al.*, 1993) (Figure 1.12).

**Figure 1.11. Quinolone-DNA binding model for gyrase inhibition.(Figure taken from Shen *et al.*, 1989)**

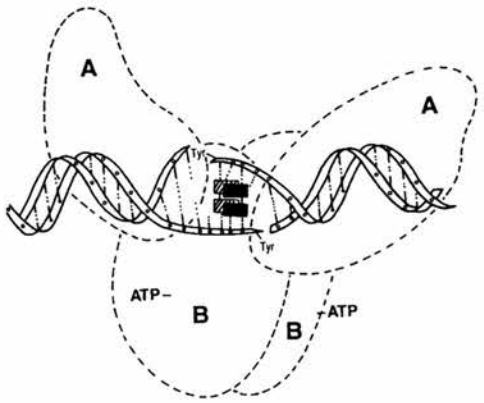
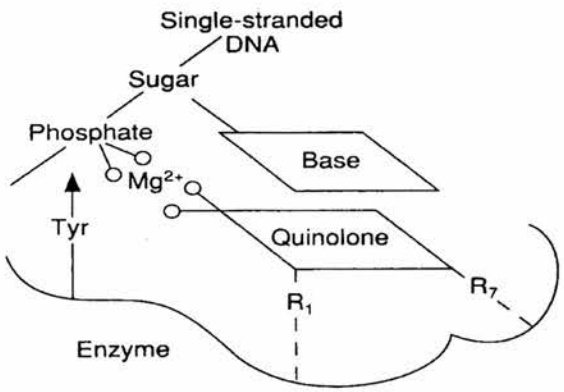



**Figure 1.12. The Quinolone-DNA binding via a $Mg^{2+}$ bridge (Figure taken from Palumbo *et al.*, 1993)**



Critchlow and Maxwell demonstrated that DNA gyrase mutants which have tyrosine - 122 replaced with either phenylalanine or serine can bind quinolone drugs. It implies that DNA cleavage is not crucial for drug binding (Critchlow and Maxwell, 1996), thus contesting the model proposed by Shen *et al.* (Shen *et al.*, 1989). The precise mechanism of quinolone, topoisomerase and DNA binding is still unknown. A high-resolution structure study, which is extremely difficult by the large size of DNA gyrase (~400 kDa) is required.

It has been demonstrated that inactivation of DNA gyrase severely inhibits DNA synthesis and results in cell death. In contrast, DNA synthesis is not significantly affected in topoisomerase IV mutants despite having defects in chromosome partitioning (Adams *et al.*, 1992; Khodursky *et al.*, 1995). This is validated in nature where *Mycobacterium* spp and *H. pylori* (Hooper, 1998) do not possess topoisomerase IV, suggesting that either DNA gyrase or an equivalent of the enzyme compensate for the absence of topoisomerase IV.

Topoisomerase IV has found to be inhibited by quinolones but it is not as sensitive to the drugs as DNA gyrase (Hoshino *et al.*, 1994; Peng and Marians, 1993). It is thought that DNA gyrase is a primary target of quinolones as its superior topoisomerase activity. However, in Gram-positive bacteria, such as *S. aureus* and *S. pneumoniae*, topoisomerase IV appears to be a primary target (Munoz and De La Campa, 1996; Ng *et al.*, 1996).

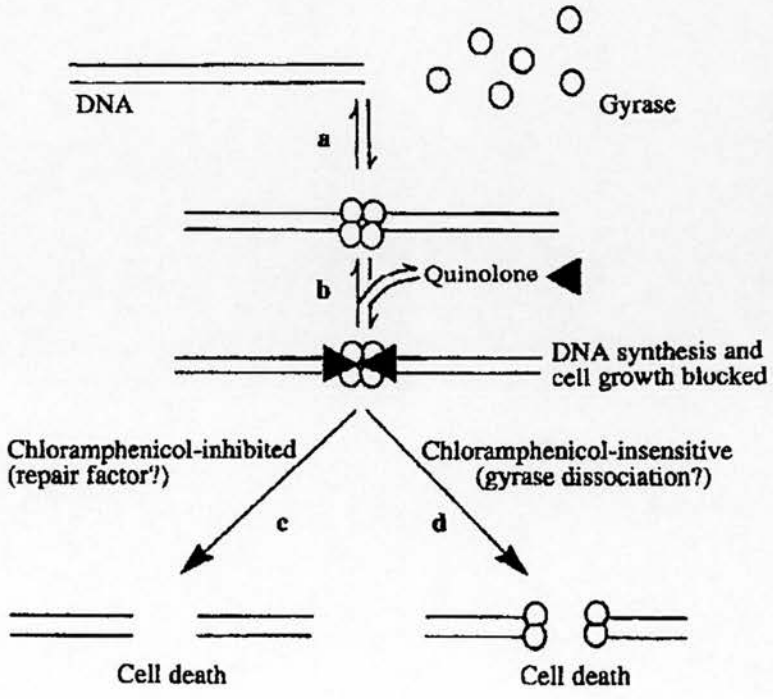
### 1.10.5 Mechanism of Quinolone Action

Quinolones target gyrase or topoisomerase IV by trapping the enzymes on DNA as ternary drug-enzyme-DNA complexes (Drlica, 1999), containing broken DNA. DNA fragmentation is described as gyrase-mediated strand passage (Figure 1.13). Gyrase wraps around DNA and produces a pair of single-stranded breaks. The quinolones trap the DNA-gyrase complex after DNA cleavage. These cleavable complexes block replication fork movement (Hiasa *et al.*, 1996), leading to inhibition of DNA synthesis and subsequent cell death. The ternary complex formation is crucial for cytotoxicity. The complexes are reversible and the DNA strand can be religated. Hiasa *et al.* purposed that the collision of a replication fork with a topoisomerase-quinolone-DNA complex converts the cleavable complex to an unreversible form and a double-strand break is probably generated by subsequent denaturation of the topoisomerase, resulting from an aborted attempt at repair of the complex, signaled by the termination of DNA replication (Hiasa *et al.*, 1996). Figure 1.12 shows the intracellular action of quinolones that mediate cell death.

Chen et al showed that the lethal effects of oxolinic acid, releasing of DNA breaks and cell death, were blocked by chloramphenicol, an inhibitor of protein synthesis. It is suggested that synthesis of additional protein activity is required to release the DNA ends from the quinolone-gyrase-DNA complexes. Although, chloramphenicol is very effective at blocking the lethal effect of oxolinic acid, it has only partial effect on ciprofloxacin, a potent fluoroquinolone. This indicates that the second lethal mode arises

from dissociation of gyrase subunits, constraining DNA ends in ciprofloxacin-gyrase-DNA complexes. Furthermore, it was demonstrated that ciprofloxacin has two intracellular targets, DNA gyrase and topoisomerase IV, and DNA gyrase is the major quinolone target in *E. coli* (Chen *et al.*, 1996).

**Figure 1.13. Schematic representation of events involved in quinolone-mediated cell death.** Gyrase and DNA interact (a) in such a way that 4-quinolone compounds can trap (b) a reaction intermediate that blocks DNA synthesis and cell growth. The DNA ends can be released by two pathways, leading to cell death. Pathway c is inhibited by chloramphenicol. It is postulated that pathway c is mediated by a repair factor, removing quinolone–gyrase complexes from DNA. At high concentrations of quinolones, the DNA ends can be released by a chloramphenicol-insensitive pathway (d). This pathway is thought to involve in DNA gyrase subunit dissociation (Chen *et al*, 1996).



## 1.10.6 Mechanism of Quinolone Resistance

The quinolones are a group of synthetic antibiotics, including nalidixic acid and fluorinated quinolones. Different mechanisms are involved in the development of quinolone resistance. Alterations in DNA gyrase and topoisomerase IV and mutations affecting the accumulation of quinolones including either reduced expression of porins or lipopolysaccharide, or active efflux are associated with the mechanism of resistance.

### 1.10.6.1 Mutations in Quinolone Targets

Quinolones target the enzyme DNA gyrase in the bacterial cell (Yoshida *et al.*, 1988). Sequencing analysis of DNA from many bacterial species has demonstrated that mutations near the active site (Tyr 122 in *E. coli*) of the A subunit of DNA gyrase confer resistance. The mutations are located in a region of *gyrA* known as the quinolone resistance-determining region (QRDR) at the amino acid terminus between amino acid 67 and 106, which is highly conserved within bacterial species. A single amino acid substitution at positions 67, 81, 83, 84, 87 or 106 has been involved in the resistant mechanism. Alterations in the QRDR have been reported in a variety of bacterial species such as *E. coli, Salmonella* spp., *Acinetobacter* spp, *S. aureus*, *S. pneumoniae* and *N. gonorrheae*. Mutations of Serine 83 and Aspartic acid 87 in GyrA of *E. coli* confer high-level quinolone resistance. Amino-acid substitutions at Serine 83 to a hydrophobic amino acid normally confer more resistance than does mutation at Aspartic acid 87.

Alterations in the *gyrB* gene have been reported to contribute to resistance in some isolates and these changes are associated with a specific QRDR (Nakamura *et al.*, 1989). Mutations at position 426 and 447 in the *gyrB* gene also confer resistance. The drug-enzyme interactions are involved in gyrase sensitivity to quinolones (Yoshida *et al.*, 1991). The frequency of *gyrB* mutations, however, is much lower in comparison with the frequency of *gyrA* mutations.

Mutations in topoisomerase IV are involved in quinolone resistance. It has been demonstrated that topoisomerase IV is a secondary target of the quinolone in Gram-negative bacteria such as *E. coli* and *A. baumannii* (Khodursky *et al.*, 1995; Vila *et al.*, 1997). Topoisomerase IV appears to be the primary target of the quinolones in Gram-positive bacteria (Ferrero *et al.*, 1995; Tankovic *et al.*, 1996).

Resistance to quinolones most commonly involves alterations of the DNA gyrase and topoisomerase IV, however, other mechanisms including decreased drug permeability or active efflux have been linked to the mechanism of resistance.

### 1.10.6.2 Reduction of Quinolone Accumulation

Quinolones enter the bacterial cell wall either directly through the lipid bilayer (hydrophobic quinolones such as nalidixic acid) or through water filled protein pores, the porins (hydrophilic compounds such as ciprofloxacin, ofloxacin) (Hirai *et al*, 1986).

Quinolone resistance due to reduced drug accumulation involves reduced expression of porin proteins and overexpression of active efflux system.

OmpF and OmpC are two major outer membrane porins in *E. coli*. In *E. coli*, OmpF porins are the major gate for the entry of hydrophilic quinolones. Mutations in *ompF* have less effect on the penetration of hydrophobic compounds. Since OmpF is an unspecific porin, several unrelated drugs such as tetracycline, chloramphenicol and some β-lactam, can enter into the cell by this porin. Loss of OmpF porin leads to resistance to all these antibiotics. In addition to mutations in the *ompF* gene, a decreased in OmpF porin appears to be linked to the alterations in unrelated loci such as *mar*RAB *and sox*RS loci (Ariza *et al.*, 1994; Miller *et al.*, 1994).

Another mechanism involved in quinolone resistance is the expression or overexpression of efflux system that remove antimicrobial agents from the cell. Efflux of quinolones is an energy-requiring transport process. NorA efflux pump are associated with quinolone resistance in *S. aureus*. Mutations in the promotor region of the *norA* confer resistance to hydrophilic fluoroquinolones such as ciprofloxacin and norfloxacin (Kaatz *et al.*, 1993; Ng *et al.*, 1994). PmrA, the efflux pump in *S. pneumoniae*, involves in fluoroquinolone resistance (Gill *et al.*, 1999). Inactivation of *pmrA* gene leads to increased susceptibility to norfloxacin and ciprofloxacin but has no effect on sparfloxacin and moxifloxacin (Gill *et al.*, 1999). Overexpression of the pump appears to contribute to the mechanism of fluoroquinolone resistance.

### 1.10.7 Resistance to Fluoroquinolones in *H. pylori*

Fluoroquinolones are not routinely included in current regimens used to treat *H. pylori* infection as they show a significant decrease of their activity at low pH. Resistance to fluoroquinolone (MIC $\geq$ 4mg/L) was found in the clinical trial that examine the efficacy of ciprofloxacin in the treatment of *H. pylori* infection (Moore *et al.*, 1995). Mutations in the QRDR of the *gyrA* gene, encoding the A-subunit of DNA gyrase, confer ciprofloxacin resistance. Moore *et al.* demonstrated that resistance were associated with four groups of point mutations in the *gyrA* gene with substitutions at amino acid 87, 88 and 91 and a double amino acid substitution at position 91 and 97 (Table 1.5). The most common mutation in *H. pylori* was at Aspartic acid-91 (Asp-87 in *E. coli*) whereas a substitution at Serine-83 is the most frequently reported in some other bacteria such as *E. coli* and *C. jejuni*.

**Table 1.5. Mutations in the QRDR of *gyrA* in *H. pylori* (Moore *et al.*, 1995)**

| Amino acid changes | Comparable change in *E. coli* | MIC (mg/L) |
|---|---|---|
| **None** | - | 0.0625-0.25 |
| **Asn-87 to Lys** | Ser-83 to Ala | 8 |
| **Ala-88 to Val** | Ala-84 to Pro | 4 |
| **Asp-91 to Gly, to Tyr or to Asn** | Asp-87 to Val or to Asn | 4-8 |
| **Asp-91 to Asn and Ala-97 to Val** | Asp-87 to Val or to Asn | 8 |

## 1.11 Aims of this thesis

1. To determine the antibiotic susceptibility patterns of *H. pylori* strains isolated in Scotland.
2. To determine the optimal conditions for metronidazole susceptibility testing
3. To investigate the mechanism of fluoroquinolone resistance in the laboratory mutants and in clinical isolates.
4. To investigate the mechanism of macrolide resistance in clinical isolates.
5. To investigate the role of *rdxA* gene in metronidazole resistance in clinical isolates.
6. To investigate the DNA gyrase activity in *H. pylori* by cloning, expression and purification of GyrA and GyrB.

# Chapter 2 Materials and Methods

## 2.1 Bacterial Strains and Plasmids

One hundred and ten isolates of *H. pylori* were isolated from 454 gastric biopsies from patients undergone endoscopy at the Royal Infirmary between 1999-2001 (Dr. R. Heading and F. Paton, Royal Infirmary, Edinburgh). All strains were single isolates from individual patients and used for antibiotic susceptibility patterns and mechanisms of resistance studies. A culture collection of 20 clinical isolates of *H. pylori* collected in 1997 was received from Dr. A. Alkout (Infection and Immunity laboratory, University of Edinburgh) and were used for metronidazole susceptibility testing study and selection of fluoroquinolone-resistance mutants. *E. coli* BL21-DE3 pLysS and *E. coli* TopF', used for cloning and overexpression of GyrA and GyrB, were supplied by Invitrogen (USA). Standard strains used in this study were *H. pylori* NCTC 11637, supplied by the Central Public Health Laboratory (Colindale), and *H. pylori* NCTC 11916, received from Dr. A. Alkout.

The plasmids used for overexpression of GyrA and GyrB was pCR T7/NT-TOPO, supplied by Invitrogen (USA). pBR322, supplied by GibcoBRL (Life Technology Ltd., Paisley, UK) was used for supercoiling activity experiment.

## 2.2 Antimicrobial Agents

Antimicrobial agents, manufacturers and solubility information are listed in Table 2.2. Fresh stock solutions of all antibiotics were prepared on the day of use.

**Table 2.1 Antimicrobial agents and their solvents**

| Antimicrobial agent | Solvent | Manufacturer/Supplier |
|---|---|---|
| Ciprofloxacin | Water | Bayer plc |
| Chloramphenicol | Water/Ethanol | Sigma-Aldrich |
| Ampicillin | Water | Sigma-Aldrich |
| Vancomycin | Water | Eli Lilly and Company |
| Cefsulodin | Water | Sigma-Aldrich |
| Trimethoprim | Water/0.5M HCl 10% of final volume | Sigma-Aldrich |
| Amphotericin B | Water | E.R. Squibb & Son Ltd. |

## 2.3 Chemicals, Buffers and Enzymes

All chemicals are purchased from Sigma-Aldrich Company Ltd. (Poole, UK) unless otherwise stated. Acetic acid and hydrochloric acid were purchased from BDH (Poole, UK). Saline was made up with 0.85% (w/v) NaCl and sterilized before used by

autoclaving. Buffers were made according to data for Biochemical Research (1974). All PCR reagents and restriction enzymes were supplied by Promega unless otherwise stated.

## 2.4 Media

### 2.4.1 Culture Media

Media used in this study were Columbia agar, Brain Heart Infusion Broth and Muller Hinton agar, supplied by Oxoid (Basingstoke, UK). LB (Luria-Bertani) broth and LB agar were supplied by GibcoBRL (Life Tecnologies Ltd., Paisley, UK). All media were made up with distilled water according to the manufacturer's instructions. Prior to use all media were sterilized by autoclaving at 121°C and 15psi for 15 minutes to destroy all vegetative cells and spores.

### 2.4.2 Transport Media

Gastric biopsy samples were transported on ice to the laboratory in 0.5ml sterile saline. The samples were kept on ice and processed in the laboratory within 4 hours.

## 2.5 Bacterial Identification

Gastric biopsy samples from the antrum were obtained from the patients, who consented to take part in this study. A single antral biopsy specimen was taken from each patient

and kept in sterile saline on ice before processed in the laboratory. Gastric biopsies were ground and grown on selective and non-selective media. Columbia agar supplemented with 7% horse blood was used as non-selective media whereas Columbia agar, containing 7% horse blood, 10mg/L vancomycin, 5mg/L trimethoprim, 5mg/L amphotericin B and 5mg/L cefsulodin was used as selective media. The plates were incubated in anaerobic jar under microaerophilic conditions (6% $O_2$ and 10% $CO_2$) provided by gas generating kit (Campylobacter system BR60, Oxoid, Unipath Ltd., Basingstoke, UK) at 37°C for 3-7 days. The strains were identified as *H. pylori* by gram stain, colony morphology and positive urease, catalase and oxidase activity. All *H. pylori* strains were stored at –70°C in brain heart infusion broth containing 20% glycerol and were subcultured onto Columbia agar plates supplemented with 7% horse blood before use.

## 2.6 Antimicrobial Susceptibility Testing

### 2.6.1 Minimum Inhibitory Concentrations (MICs)

The colonies on the Columbia agar, supplemented with 7% horse blood were harvested and suspended in brain heart infusion broth to the density equal to that of No.3 MacFarland Standard to give $10^9$ cfu/ml. Minimum inhibition concentrations (MICs) were performed on Columbia agar, supplemented with 7% horse blood. A multipoint inoculator (Denley, Billinghurst, Surrey) was used to inoculate the tested organisms onto Columbia blood agar, containing serially diluted antibiotics. The plates were incubated

under microaerophilic conditions at 37°C for 72 hours. The MIC was determined to be the lowest concentration with no growth.

### 2.6.2 Epsilon Test (E-test)

The E-test (AB Biodisk, Cambridge, UK) comprises a plastic strip embedded with an antibiotic gradient and employed a more stringent and convenient method for determining susceptibility of bacteria. The colonies on the Columbia agar, supplemented with 7% horse blood were harvested and suspended in brain heart infusion broth to the density equivalent to No.3 MacFarland Standard turbidity. The suspensions were flood-seeded onto Columbia agar containing 7% horse blood. The plastic strips were impregnated on the surface of the agar plates. The plates were incubated for 72 hours at 37°C under microaerophilic conditions. For metronidazole susceptibility testing only, the plates were pre-incubated under anaerobic conditions for 24 hours, followed by microaerophilic conditions for 72 hours. The manufacturer's instructions were followed in the interpretation of the results.

## 2.7 Selection of Fluoroquinolone-Resistant Mutants

Stepwise selection of ciprofloxacin-resistant mutants was performed by plating approximately $10^8$cfu of *H. pylori* onto Columbia blood agar containing increasing concentrations of ciprofloxacin. After 72 hours of incubation at 37°C under

microaerophilic conditions, first-step ciprofloxacin-resistant mutants were selected. Second-step mutants were obtained by plating the first-step mutants onto Columbia blood agar containing increasing concentrations of ciprofloxacin. Third-step mutants were obtained in the similar manner to the second-step mutants. MICs were determined by E test and agar dilution on Columbia agar containing 7% horse blood.

## 2.8 Preparation of DNA for PCR

Chromosomal DNA was extracted for PCR by using a Puragene DNA isolation kit (Gentra system kit, USA) according to the manufacturer's instructions.

## 2.9 Phenol:Chloroform:Isoamyl Alcohol DNA Extraction

Phenol:chloroform:isoamyl alcohol (25:24:1) were used to cleanup pBR322 for supercoiling assay. Sample containing pBR322 was mixed gently with equal volume of phenol/chroloform/isoamyl alcohol. The upper aqueous layer was removed to the new tube after centrifugation at 13,000g for 5 minutes. An equal volume of ice-cold isopropanol was added and mixed. The DNA was pelleted by centrifugation at 13,000g for 10 minutes. The supernatant was discarded and the pellet was dried and resuspended in distilled water.

## 2.10 Mutation Detection by the Polymerase Chain Reaction (PCR)

Mutations in the QRDR of *gyrA*, 23S rRNA and *rdxA* were detected by PCR amplification and DNA sequencing. The primers used for each PCR are shown in Table 2.3. All reactions were prepared in 100 µl volumes in a Techne Cyclogene Thermal cycler (Cambridge Biosciences, Cambridge). The products were analysed by agarose gel electrophoresis.

### 2.10.1 Amplification of the QRDR of *gyrA*.

Oligonucleotides primers 5'AATTAGGCCTACTTCCAAAGTCGCTTACA-3' and 5'-TCTTCACTCGCCTTAGTCATTCTGGC-3'(Moore et al, 1995) were used to amplify a 238bp fragment. Reactions comprised 1xPCR buffer, 1.5mM $MgCl_2$, 200µM each dNTP, 0.25µM of each primer (Oswel DNA services Ltd., Southampton), 2U of *Taq* DNA polymerase. Cycling parameters were as follows: i) one cycle of 94 °C for 60s; ii) 35 cycles of 94°C for 60s, 55 °C for 30s, 72 °C for 60s, iii) one cycle of 4 °C for 24 hours.

### 2.10.2 Amplification of the Peptidyltranferase Region of the 23S rRNA

The 23S rRNA primers were derived from the sequence published by Occhialini *et al.* (1997). Oligonucleotides primers 5'-CCACAGCGATGTGGTCTCAG-3' and 5'-

CTCCATAAG AGCCAAAGCCC-3' (Occhialini *et al*, 1997) were used to amplify a 425bp fragment. Reactions consist of 1xPCR buffer, 1.5mM $MgCl_2$, 200μM each dNTP, 0.25μM each primer (Oswel), 2U of *Taq* DNA polymerase. Cycling parameters were as follows: I) one cycle of 94 °C for 60s; ii) 35 cycles of 94°C for 60s, 54 °C for 60s, 72 °C for 60s, iii) one cycle of 72 °C for 10 minutes, iv) one cycle of 4 °C for 24 hours.

.

### 2.10.3 Amplification of *rdxA*

Oligonucleotides primers 5'ATGGGTTGCTGATTGTGGTTTATGG3' and 5'CGTTG AAAACACCCCTAAAAGAGCG-3' (Pual *et al*, 1995) were used to amplify a 947bp fragment of the *rdxA* gene. Reactions consist of 1xPCR buffer, 1.5mM $MgCl_2$, 200μM each dNTP, 0.25μM of each primer (Oswel), 2U of *Taq* DNA polymerase. Cycling parameters were as follows: i) one cycle of 94 °C for 60s; ii) 35 cycles of 94°C for 60s, 55 °C for 60s, 72 °C for 60s, iii) one cycle of 72 °C for 10 minutes, iv) one cycle of 4 °C for 24 hours.

.

## 2.11 Analysis of DNA by Gel Electrophoresis

DNA was visualised by electrophoresis on 1-1.5% agarose gel (Gibco BRL, Paisley, UK) in TAE buffer [40mM Tris-acetate (pH8.0), 2mM EDTA]. Electrophoresis was performed on horizontal gels utilising a minisubcell GT (Bio-Rad, Hemel, Hemstead UK). The DNA samples were mixed with loading buffer [0.25%(w/v) bromphenol blue, 0.25% (w/v) xylene cyanol, 30% (w/v) sucrose] at a ratio 5:1 prior to gel loading.

Samples were electrophoresed at a constant voltage between 70 and 90V (Powerpac 300, Bio-Rad, UK) for a time length depending on DNA fragment size (30-50 min). Samples were electrophoresed alongside a suitable molecular weight marker, generally 100bp ladder (Promega), 1kb ladder and lambda *Hin*dIII DNA (Promega).

## 2.12 Staining and Visualisation of DNA

Following electrophoresis, gels were stained in a 0.5μg/mL ethidium bromide solution and visualised on a UV trans-illuminator (UV products, Cambridge). The Bio-Rad GelDoc 20000 system (BioRad) was also used to visualise and photograph gels.

## 2.13 DNA Quantification

A rapid method to estimate DNA concentration was perform by electrophoresising 1μl of DNA sample alongside 2μl of 0.5μg Lambda/*Hin*dIII DNA (Promega), each band of which is known to contain a specified DNA concentration. The gel was stained in ethidium bromide solution. The intensity of the sample bands were compared with Lambda/*Hin*dIII DNA bands to estimate the concentration of DNA.

## 2.14 Sequencing of PCR Products

The PCR products were purified by using a QIAquick PCR purification kit (Qiagen, Germany) according to the manufacturer's instructions. The purified products were

eluted from the column into 30 μl of elution buffer to concentrate the DNA to ensure that it contained 30-90ng DNA to run on an automatic sequencer. The PCR primers used for PCR amplification were also used for DNA sequencing, with 3.2 pmole of primer per sequencing reaction. All the sequences were processed in the Department of Haematology, Royal Infirmary of Edinburgh. The DNA sequence was determined by chain termination method developed by Sanger *et al* (1997). Individual PCR fragments were set up in the ready reaction format for fluorescence based on dideoxy cycle sequencing (PE Applied System, UK). DNA sequences were analysed by Chromas Version 1.56 software and compared to the published sequences by using the BLAST online search (http://www.ncbi.nlm.nih.gov/ BLAST). The amino acid sequences were translated using EXPASY translate website (http:www.expasy.ch.tools/DNA/html). Multi-alignment of DNA and amino acid sequences were performed with the online website multalin (http://www.toulous.inra.fr/multalin.html).

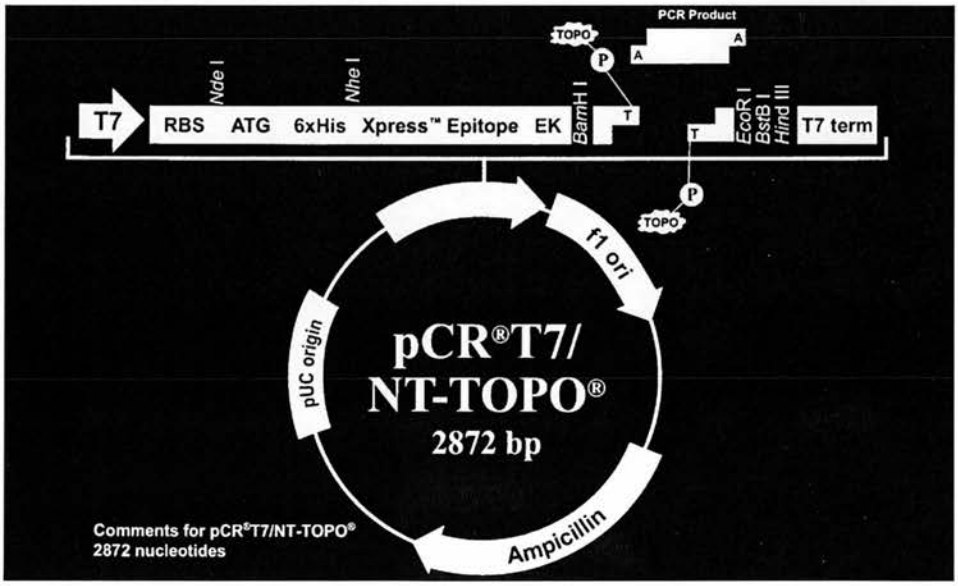
## 2.15 PCR Cloning and Expression of GyrA and GyrB

PCR cloning and expression of GyrA and *gyrB* were carried out with the pCR T7 TOPO TA Expression kit (Invitrogen, USA), according to the manufacturer's instructions All reagents, competent cells and plasmids were supplied by the kit unless otherwise stated.

## 2.15.1 Primer Design

The forward primers hybridised at the beginning of the genes and the reverse primers were designed to hybridise downstream of the stop codon and the forward primers hybridised at the beginning of the genes. The actual sizes of *gyrA* and *gyrB* are 2478bp and 2322bp, respectively. The PCR products, amplified by *Taq* polymerase, had 3' A-overhangs, which were able to ligate to the pCR-T7/NT vector, a T7 expression vector containing 3' T-overhangs (Figure 2.1). The proteins overexpressed by this vector contained His-tagged at the N terminal, thus enabling the expressed protein to be purified by affinity chromatography.

**Figure 2.1 pCR-T7/NT vector map**



### 2.15.2 Amplification of Complete Sequences *gyrA* and *gyrB*

The *gyrA* and *gyrB* primers were derived from the sequences of *H. pylori* 26695 from GenBank (Accession No. AE000583), by using Primer 3 software (http:www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi). Chromosomal DNA was obtained from *H. pylori* NCTC 11637 as previously described. Two oligonucleotide primers, 5'-ATGCAAGATAATTCAGTCAATGAA-3' and 5'-TTCAAGCGGTACGA AATTAAAAA-3' were used for *gyrA* amplification. Oligonucleotide primers 5'-ATGC AAAATTACCAGAGCCATAGT-3'and 5'-CCCCTAAATTCACGCTCTCT-3' were used for *gyrB* amplification. Temperature cycling was performed on a GeneAmp PCR system 9600 (Perkin-Elmer).

For *gyrA* amplification, a PCR reaction (100µl) of 1xPCR buffer, 2mM $MgCl_2$, 200uM each dNTP, 0.25µM of each primer, 2U of *Taq* DNA polymerase in MilliQ water were cycled at the following parameters: 2 minutes at 94°C followed by 30 cycles of 94°C for 1 minute, 55 °C for 1 minute and 72 °C for 3 minutes with the final extension of 72 °C for 10 minutes producing a product of 2590bp.

For *gyrB* amplification, a PCR reaction (100µl) of 1xPCR buffer, 3mM $MgCl_2$, 200uM each dNTP, 0.25µM of each primer, 2U of *Taq* DNA polymerase in MilliQ water were cycled at the following parameters: 2 minutes at 94°C followed by 30 cycles of 94°C for 1 minutes, 55 °C for 1 minutes and 72 °C for 3 minutes with the final extension of 72 °C for 10 minutes producing a product of 2380bp.

### 2.15.3 Cloning of PCR Products

PCR products of *gyrA* and *gyrB* were excised and purified with a Gel Extraction Kit (Qiagen) according to the manufacturer's instruction. The *gyrA* and *gyrB* genes were separately cloned into pCR-T7/NT vector (Invitrogen), which encodes an N-terminal 6xHis-G tag thus enabling the expressed protein to be purified by affinity chromatography on a ProBond resin column (Invitrogen). The recombinant plasmids were transformed into competent *E. coli* TOP10F'cells and selected on LB agar containing 100mg/L ampicillin. Plasmids from the positive clones were extracted and purified with Wizard Plus SV Minipreps DNA purification system (Promega), according to the manufacturer's instruction. The plasmids were further analysed by *Hin*dIII restriction analysis and DNA sequencing.

### 2.15.4 Restriction Analysis of Recombinant Plasmids

Plasmids of the transformants were extracted and analysed for the orientation of the inserts by *Hin*dIII. A 4µl of plasmid was added to 2 µl restriction enzyme buffer (6mM Tris-HCl, 6mM Mg $Cl_2$, 100mM NaCl, 1mM DTT, pH 7.5), 0.2µl bovine serum albumin (10mg/ml) and 5U (Promega), final volume 20 µl. The reaction mixtures were incubated at 37C for 4 hours and run on a 1.5% agarose gel and visualised as previously described. If the restriction sites are cut with this enzyme, the plasmid containing *gyrA* gives the products of 4459bp, 537bp and 450bp and that of *gyrB* was 3671bp, 1023bp, 480bp and 90bp.

### 2.15.5 Sequencing of Plasmids Containing *gyrA* and *gyrB* Inserts

The orientation and its insertion in frame with the vector's 6xHis tag were verified by DNA sequencing, using the vector's forward and reverse primers and primers chosen from the internal sequences of the *gyrA* and *gyrB* genes. The plasmids were sent to the Department of Haematology, Royal Infirmary of Edinburgh for DNA sequencing. The sequencing primers are shown in Table 2.2

**Table 2.2 Oligonucleotide primers used for DNA sequencing for *gyrA***

| Primer | Sequence |
|---|---|
| **HP-WA2** | 5'-TTT AGT CAA TGG GGC TAA TGG-3' |
| **HP-WA3** | 5'-CGC GAT GAG TGA AAT TGT CTT-3' |
| **HP-WA4** | 5'-CTC CAA GGC GCA CTG AAA T-3' |
| **HP-WA5** | 5'-GAA AAA TGG CGT GGT GAA G-3' |
| **T7** | 5'-TAA TAC GAC TCA CTA TAGGG-3' |
| **T7 Reverse** | 5'-TAG TTA TTG CTC AGC GGTGG-3' |

**Table 2.3 Oligonucleotide primers used for DNA sequencing for *gyrB***

| Primer | Sequence |
|---|---|
| **GyrB-F1** | 5'- ATGC AAAATTACCAGAGCCATAGT-3' |
| **GyrB-F2** | 5'-CGT GAT GGA AGT CGT TGA ATT T-3' |
| **GyrB-F3** | 5'-GCC AGA GAG CTT ACA AGG AAA A-3' |
| **GyrB-F4** | 5'-GGT GGA TAT TGA AGG GAT TGG-3' |
| **T7** | 5'-TAA TAC GAC TCA CTA TAGGG-3' |
| **T7 Reverse** | 5'-TAG TTA TTG CTC AGC GGTGG-3' |

## 2.15.6 Overexpression of GyrA and GyrB

Plasmids of the positive clone were overexpressed in BL21(DE3)-pLysS cells. The bacteria was selected on LB broth containing 100mg/L ampicillin and 37mg/L chloramphenicol at 37 °C overnight. A 20ml of overnight culture was used to inoculate 1L of LB broth containing 100mg/L ampicillin and 37mg/L chloramphenicol at 37°C. At an OD600 of 0.5, the cells were induced with 1mM isopropyl thio-β-D-galactoside (IPTG) for 4 hours at 37°C. Cells were collected by centrifugation at 1000g for 30 minutes, resuspended in sonication buffer (20mM phosphate buffer, 500mM NaCl, pH7.8) and lysed by sonication. The cell lysate was centrifuged at 3000g for 30 minutes to obtain cell-free extracts. The lysate was passed through a 0.8μm syringe filter and was stored on ice or at -20 °C. This was then purified on an affinity column.

## 2.16 SDS-PAGE

The GyrA and GyrB proteins were separated by SDS-PAGE (Laemmli, 1970). The samples were boiled in sample buffer (62.5mM Tris-HCl pH 6.8, 0.2% SDS, 10% glycerol, 1% 2-mercaptoethanol, 0.01% bromphenol blue) for 5 minutes before loading onto a 4% stacking gel and 12% separating gel (Bio-Rad) in electrode buffer (25mM Tris-HCl pH 8.3, 192mM Glycine, 0.2% SDS). A constant voltage of 180 V was applied. Molecular weights were determined from standard bands using Precision Protein Standards (Bio-Rad) and were detected with Coomassie brilliant blue (40% v/v methanol, 10% v/v acetic acid, 0.25% w/v Coomassie blue R-250) for 45 mins and destained by destaining solution (40% v/v methanol, 10% v/v acetic acid in distilled water). All gel anlysis was carried out using Quantity One software (Bio-Rad).

## 2.17 Western Blot

The GyrA and GyrB proteins were resolved on an SDS-PAGE gel. Equilibrate the gels, membranes, filter papers and fiber pads in transfer buffer for 30 minutes. The PVDF (polyvinylidine difluoride) membranes were wetted in 100% methanol before soaking in the buffer. Pre-wetted PVDF membranes were applied to an SDS gel containing the separated proteins. The proteins were transferred while a constant voltage of 100V was applied for 1 hour in the cold room by the Trans-Blot electrophoretic transfer cell (Bio-Rad). The transfer buffer was 25mM Tris, 192 mM glycine, pH 8.3.

The blots were washed with TBS-T (20mM Tris-HCl pH7.6, 137mM NaCl, 0.1% Tween 20) for 15 minutes and then blocked with 5% skimmed milk powder in TBS-T for 1 hour at room temperature. The membranes were incubated with specific antibody to HisG conjugated with HRP at 1:5000 dilution at room temperature for 2 hours. The membranes were washed extensively with TBS-T at room temperature 4 times, 15 minutes each. The TMB substrate kit (Vector Laboratories, USA) was used to detect labelled proteins, according to the manufacturer's instructions.

## 2.18 Purification of GyrA and GyrB

GyrA and GyrB were purified by ProBond Purification System (Invitrogen). The recombinant protein with contains six tandem histidine residues at the N-terminal has a high affinity for ProBond resin. The sample was loaded onto a ProBond column, previously equilibrated with Native binding buffer (20mM sodium phosphate, 500mM NaCl, pH 7.8). The column was washed with Native binding buffer pH 6.0 (20mM sodium phosphate, 500mM NaCl, pH 6.0) until the $OD_{280}$ is less than 0.01. The protein was eluted by gradient imidazole (50mM, 200mM, 350mM and 500mM) according to the manufacturer's instruction.

## 2.19 Supercoiling Relaxed DNA Activity

DNA gyrase catalyses the ATP-dependent introduction of supercoils into relaxed DNA. Relaxed DNA was prepared by treating pBR322 DNA with DNA topoisomerase I. The supercoiling assay was carried out using relaxed DNA as a substrate.

### 2.19.1 Relaxed DNA Preparation

Standard reaction mixtures (20μl) containing 35mM Tris-HCl, pH 8.0, 72mM KCl, 5mM $MgCl_2$, 5mM DTT, 5mM spermidine, 0.01% bovine serum albumin, 0.5μg supercoiled pBR322 DNA (Gibco, BRL) and 1 Unit of topoisomerase I (Armersham Phamacia Biotech, UK) were incubated at 37 °C for 1 hour. The mixtures were then extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1). After centrifugation at 13,000g for 5 minutes, the aqueous layer was added to an equal volume of ice cold isopropanol. The DNA was pellet by centrifugation at 13,000g for 10 minutes and resuspended in 50 μl of sterile distilled water.

### 2.19.2 Assay of DNA Supercoiling

Supercoiling activities were carried out in 40μl of 35mM Tris-HCl, pH 7.5, 25mM KCl, 4mM $MgCl_2$, 2mM DTT, 1.8mM spermidine, 1mM ATP, 6.5%glycerol, 0.1mg/ml bovine serum albumin, 0.2μg relaxed pBR322 DNA. *E. coli* DNA gyrase (Sigma, UK) was used as a positive control. Serial dilutions of partially purified GyrA and GyrB were determined for the optimal activity. The samples were electrophoresed in a 1% agarose

gel for 45 minutes at 80V. The gel was stained with 0.5μg/ml ethidium bromide and visualised on a UV transluminator.

# Chapter 3 Results: Antibiotic Susceptibility of *H. pylori*

## 3.1 Introduction

*H. pylori* has been recognised as a causative agent of gastritis and peptic ulcer diseases. The potential for antimicrobial therapy in the treatment of *H. pylori* infection has resulted in the reported on in-vitro susceptibilty of *H. pylori* to antimicrobial agents. Many antibiotics have excellent in-vitro activity against *H. pylori*, however, when antibiotics are used alone, the development of resistance usually occurs. Multi-drug antimicrobial therapies including combinations of clarithromycin, metronidazole or amoxycillin and proton pump inhibitors are widely used to eradicate *H. pylori*.

The prevalence of antibiotic resistance varies between countries. It is important to update the antibiotic resistant profile of individual area. Susceptibility patterns of antibiotics against *H. pylori* have not yet been reported in Scotland. This study investigated the prevalence of antibiotic resistance of *H. pylori* and the in-vitro activity of amoxycillin, ciprofloxacin, clarithromycin, erythromycin, tetracycline and metronidazole against *H. pylori* clinical isolates.

## 3.2 Collection of Clinical Strains of *H. pylori* in Scotland

*H. pylori* isolates were obtained from antral biopsies from the 454 patients undergoing routine endoscopy at the Centre for Liver and Digestive Disorders, Royal Infirmary, Edinburgh, Scotland. The specimens were collected during the period December 1999 - March 2001. This project was a prospective study and was approved by the ethical committee. Sample collection and patient consent were a source of delay. One biopsy sample from antrum per patient was used to isolate *H. pylori*.

Of the 454 gastric biopsies, 110 *H. pylori* strains were isolated. The isolates were identified as *H. pylori* by oxidase, catalase production, rapid positive urease test and Gram-stain. The *H. pylori* isolation rate was low at 24.2% (110/454). This can be explained by the fact that biopsies were obtained from patients undergoing routine endoscopy, not only the patients with gastritis or peptic ulcer disease. Only one biopsy sample was obtained from each patient, which could also reduce the *H. pylori* isolation rate.

## 3.3 Antibiotic Susceptibility Testing

Susceptibility profiles of 110 *H. pylori* strains were determined by E-test to amoxycillin, ciprofloxacin, clarithromycin, erythromycin, tetracycline and metronidazole. The range of MICs, $MIC_{50}$, $MIC_{90}$ of amoxycillin, ciprofloxacin, clarithromycin, erythromycin, tetracycline and metronidazole against 110 clinical isolates are shown in Table 3.1, Figure 3.1 and Figure 3.2 The MICs of all strains are shown in Appendix I. The MIC

break points are >8mg/L for amoxycillin, >2mg/L for ciprofloxacin, >2mg/L for clarithromycin, >2mg/L for erythromycin, >2mg/L for tetracycline and ≥4mg/L for metronidazole.

**Table 3.1 MICs of 6 antibiotics against 110 *H. pylori* clinical isolates**

| **Antibiotic** | **$MIC_{50}$ (mg/L)** | **$MIC_{90}$ (mg/L)** | **Range (mg/L)** |
|---|---|---|---|
| Amoxycillin | <0.016 | 0.016 | <0.016-0.047 |
| Ciprofloxacin | 0.032 | 0.064 | 0.003->32 |
| Clarithromycin | <0.016 | 0.023 | <0.016->256 |
| Erythromycin | 0.023 | 0.125 | <0.016->256 |
| Tetracycline | 0.023 | 0.064 | <0.016-1 |
| Metronidazole | 0.016 | 0.75 | <0.016->256 |

**Figurer 3.1 $MIC_{50}$ and $MIC_{90}$ values of *H. pylori* against 6 antibiotics**

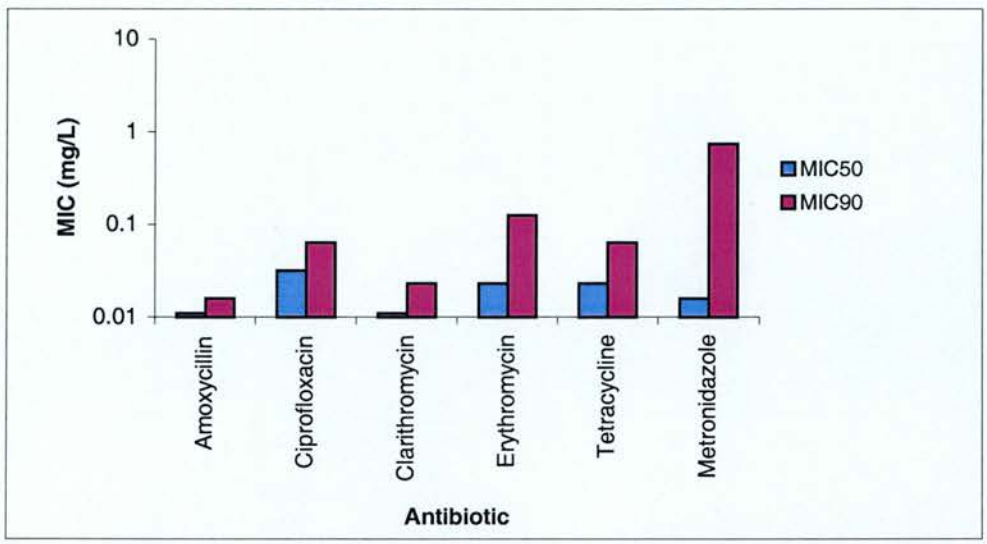
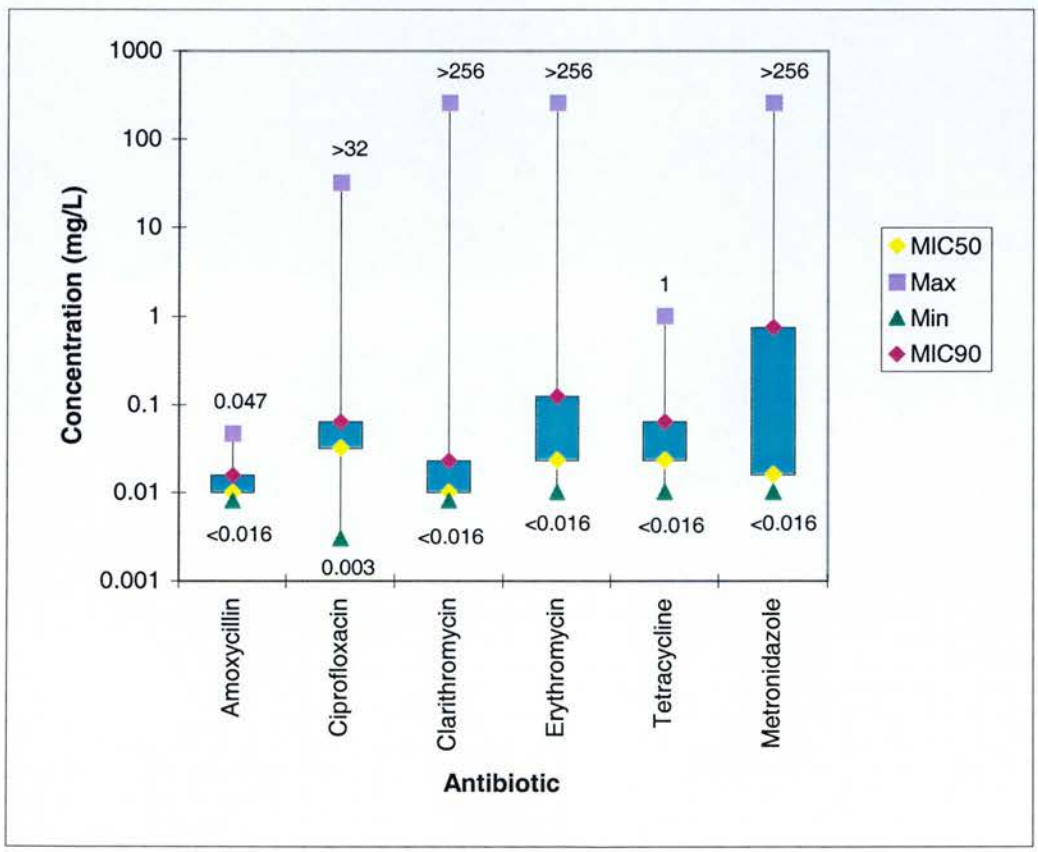



**Figure 3.2 $MIC_{50}$, $MIC_{90}$ and range of *H. pylori* against 6 antibiotics.**



Analysis of the MIC data (Table 3.1) showed that amoxycillin was the most potent antibiotic against *H. pylori* as the $MIC_{90}$ is 0.016mg/L and no resistance was found in all of these isolates. Clarithromycin ($MIC_{90}$ = 0.023mg/L) was the second most potent, followed by tetracycline and ciprofloxacin with the $MIC_{90}$ of 0.064mg/L. Metronidazole was the least active antibiotic ($MIC_{90}$ = 0.75mg/L). Erythromycin was the next least active ($MIC_{90}$ = 0.125mg/L). The $MIC_{50}$ and $MIC_{90}$ values of all antibiotics are below the breakpoints.

Figure 3.3 gives an overview of the distribution of the MICs of amoxycillin, ciprofloxacin, clarithromycin, erythromycin, tetracycline and metronidazole. For all strains, the MIC of amoxycillin was <0.064mg/L. Most of the isolates (84.5%) had very low MICs (<0.016mg/L). The distribution of the MIC peaked at <0.016mg/L and gradually declined to 0.047mg/L (Figure 3.3 a).

For ciprofloxacin, the distribution of the MICs showed a set of the sensitive strains with the MIC range 0.003 – 0.19mg/L. The peak was at 0.032mg/L and then declined from both sides. The majority of the sensitive strains (94.4%) had the MIC of ≤0.064mg/L. Two strains exhibited ciprofloxacin resistance with MICs of 16 and >32mg/L (Figure 3.3 b).

For clarithromycin, most of the strains (72.7%) had a clarithromycin MIC of <0.016mg/L (Figure 3.3 c). The distribution of the MICs had a peak at the MIC of

The susceptibility patterns of amoxycillin, ciprofloxacin, clarithromycin, erythromycin, tetracycline and metronidazole against *H. pylori* isolated in Scotland demonstrated that all antibiotics tested are active against *H. pylori* ($MIC_{90}$ range 0.016-0.75mg/L) and all isolates exhibit the $MIC_{90}$ below the breakpoints for all antibiotics. However, metronidazole and clarithromycin resistance has frequently been reported as they are widely used to eradicate *H. pylori*. Metronidazole was found to be the least potent drug tested in this study with the $MIC_{90}$ of 0.75mg/L. Metronidazole resistance was observed in 8 strains with the MIC range 4 - >256mg/L. Clarithromycin has excellent activity against *H. pylori* with the $MIC_{90}$ of 0.023mg/L. However, eight isolates (MIC range 3 - >256mg/L) were found to be resistant to clarithromycin and one isolate was intermediate resistant (MIC = 1.5mg/L). All of these isolates were cross-resistant to erythromycin. Two isolates were resistant to both metronidazole and clarithromycin, suggesting that these resistances were developed after *H. pylori* treatment with these antibiotics and antibiotic susceptibility testing should be performed for the successful eradication therapy.

<0.016mg/L and then tailed off. The sensitive strains had the MIC range 0.016 - 0.125mg/L). A cluster of clarithromycin-resistant isolates was demonstrated with the MICs in the range 1.5- >256mg/L and the distribution of the MICs was in line. The MIC breakpoint for clarithromycin is >2mg/L and the MIC of 1.5mg/L is intermediate resistant.

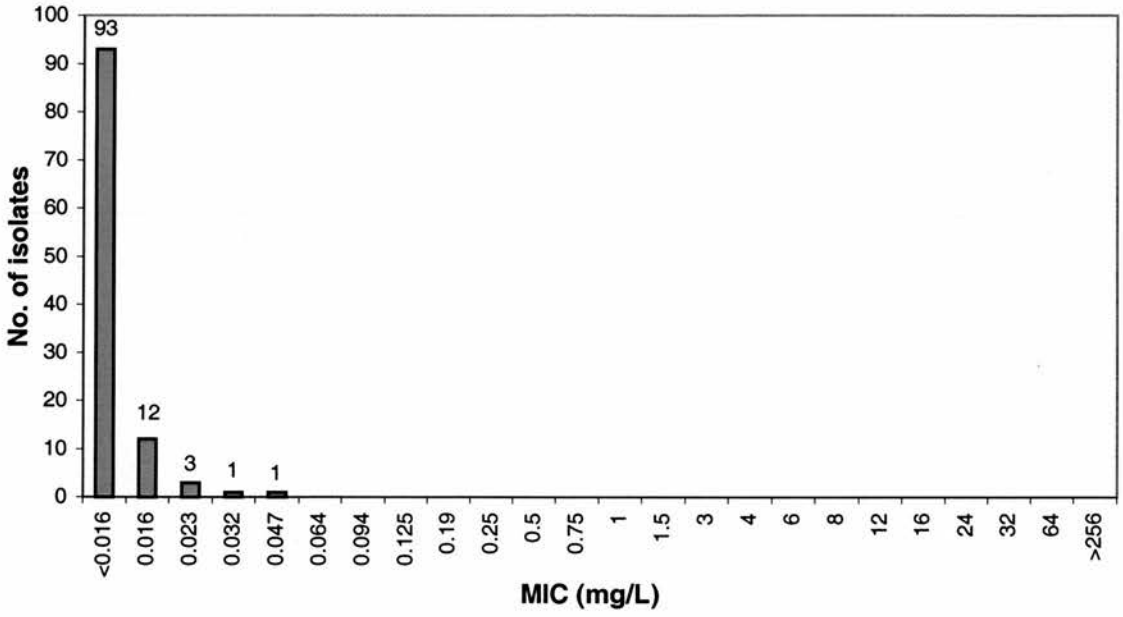
For erythromycin, the distribution of the MICs showed that all the sensitive strains had the MIC in the range of <0.016 – 0.19mg/L, with the peak at 0.023mg/L (Figure 3.3 d). Of the 100 isolates, 77 strains (77%) had the MIC of ≤ 0.032mg/L. All of the resistant strains had high-level MIC of >256mg/L.

For tetracycline, a cluster of sensitive isolates with the MIC range of <0.016 – 0.19mg/L was shown (Figure 3.3 e). The peak was at 0.016mg/L. Of the 109 sensitive strains, 91 (83.4%) had the MIC of ≤ 0.032mg/L. One isolate had the MIC of 1mg/L, which was defined as intermediate resistant.

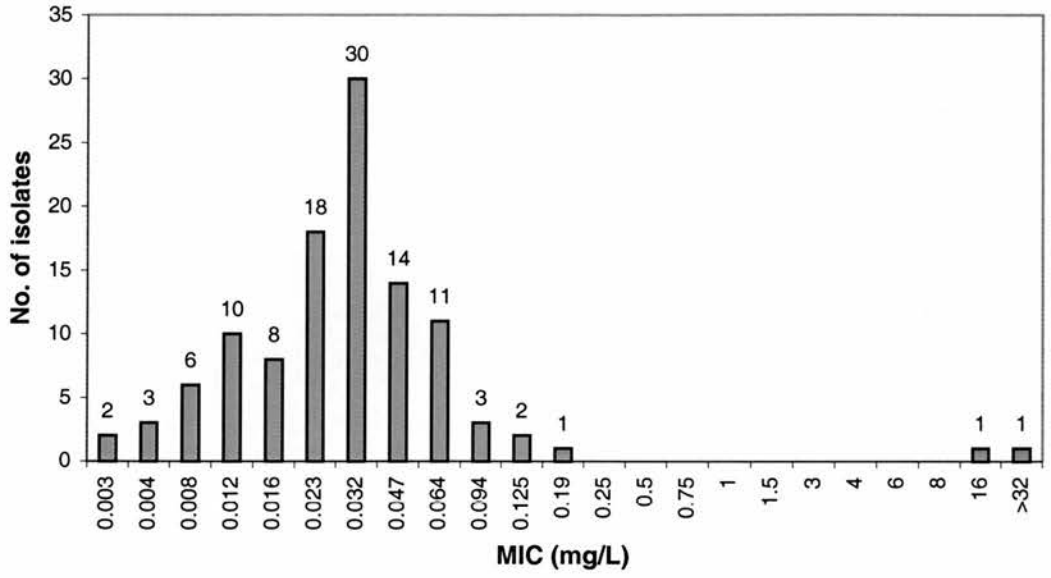
For metronidazole, most of the strains (38.2%) had the MIC of <0.016mg/L (Figure 3.3 f). The distribution of MICs showed a peak at the MIC of <0.016mg/L and then tailed off. A cluster of the sensitive strains with MICs in the range < 0.016 – 1.5mg/L) was observed. One isolate had the MIC of 4mg/L (cut-off level). A cluster of metronidazole-resistant isolates was shown, with the MIC range 12 - >256mg/L.The distribution of the MIC of this cluster was in line.

**Figure 3.3 Distribution of the MICs of 110 *H. pylori* isolates against amoxycillin (a), ciprofloxacin (b), clarithromycin (c), eryhromycin (d), tetracycline (e) and metronidazole (f).**

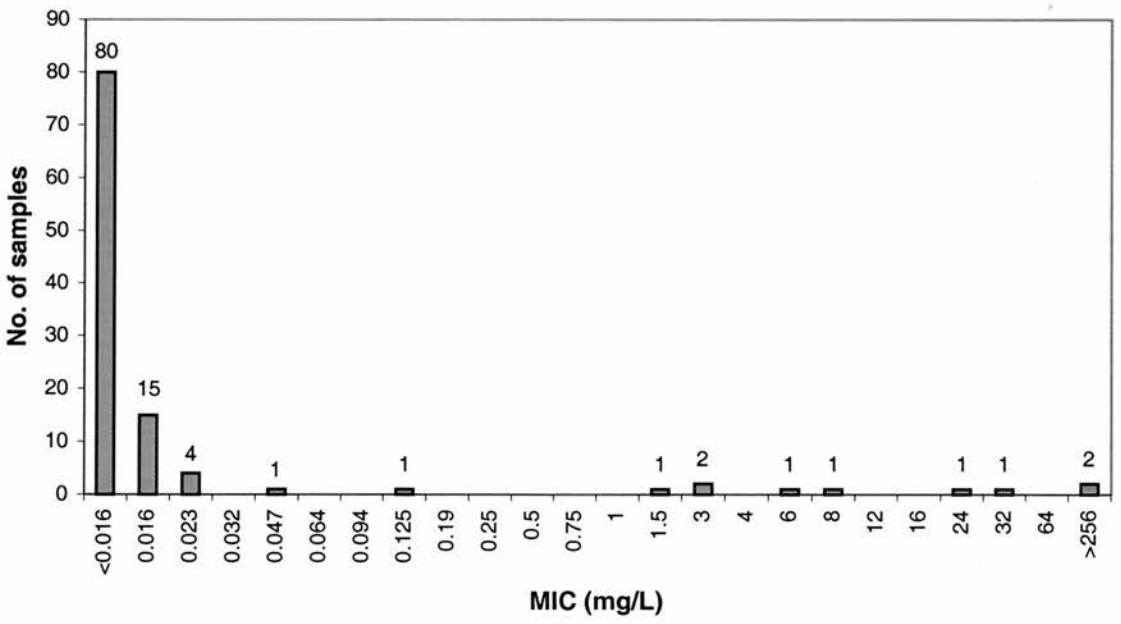
**(a) Amoxycillin**



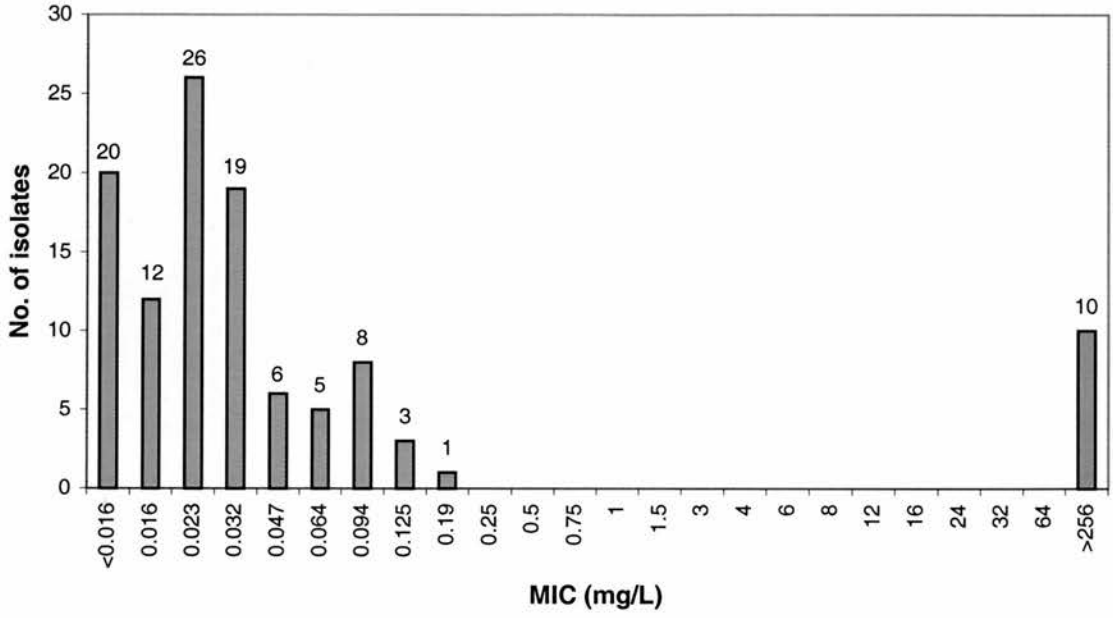
**(b) Ciprofloxacin**



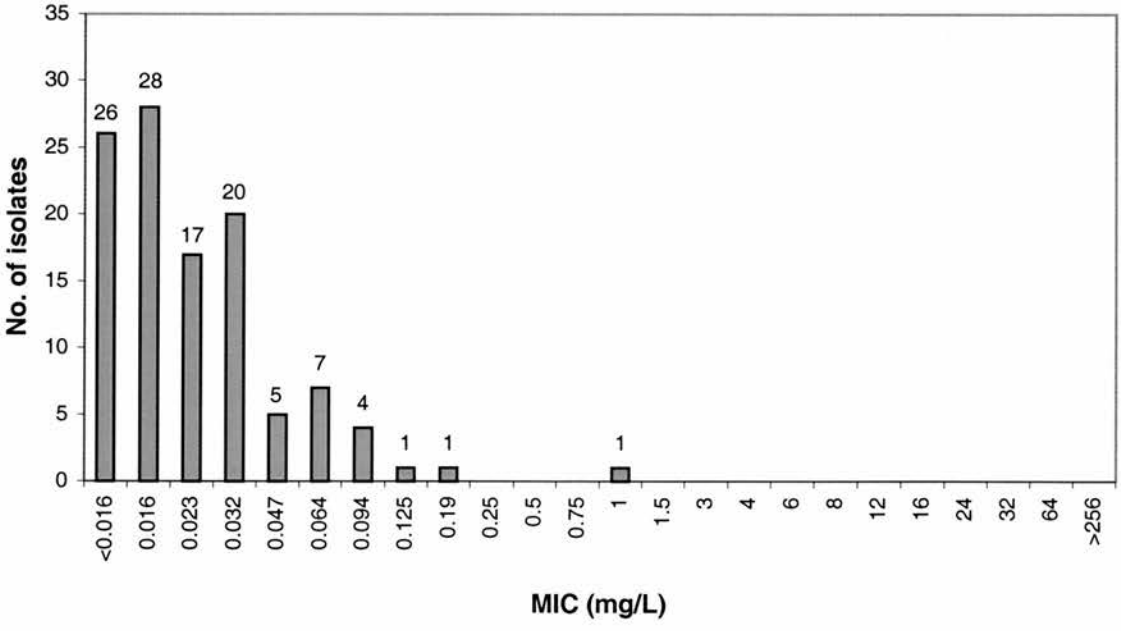

**(c ) Clarithromycin**



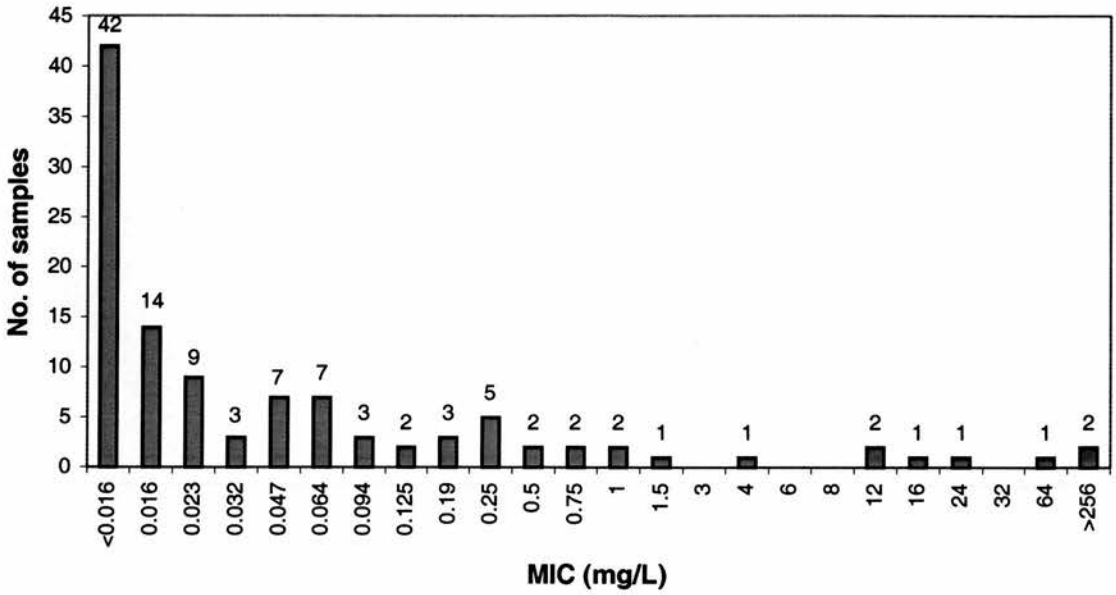
**(d) Erythromycin**




**(e) Tetracycline**



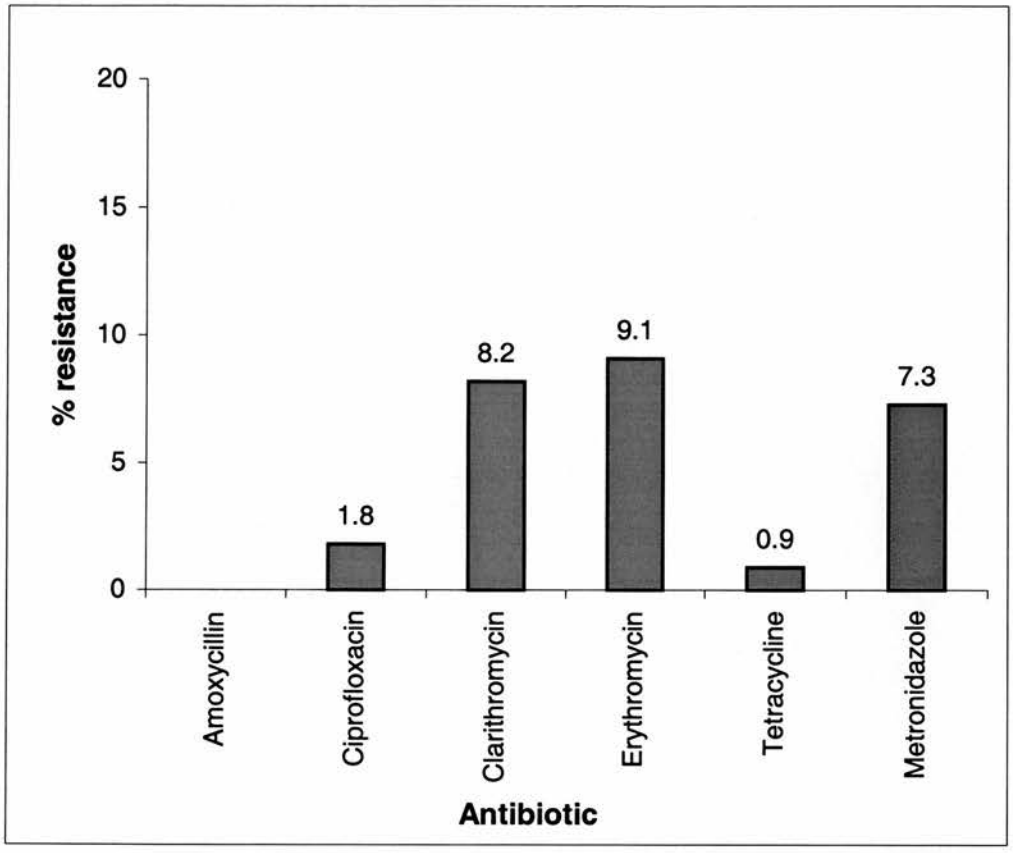
## (f) Metronidazole



## 3.4 Prevalence of Antibiotic Resistance in *H. pylori* in Scotland

Resistance to macrolides, clarithromycin and erythromycin, was found in 8.2% (9/110) and 9.1% (10/110) of the *H. pylori* strains, respectively (Figure 3.4). All clarithromycin-resistant isolates also conferred erythromycin resistance with MIC of >256 mg/L. One strain was erythromycin resistant (MIC >256mg/L) but clarithromycin-sensitive (MIC = 0.125mg/L). Resistance to metronidazole was found in 8 isolates (7.3%). Two of them were also resistant to clarithromycin and erythromycin. Ciprofloxacin resistance was found in 2 isolates (1.8%). One isolate (0.9%) was resistant to tetracycline (intermediate, MIC 1 mg/L). Amoxycillin resistance was not detected. The results from this study reveal that the prevalence of macrolide resistance in Scotland is greater than to any other antibiotics. Clarithromycin is a potent antibiotic widely used in the treatment regimen. Resistance to clarithromycin will, therefore, affect the cure rate and account for treatment failure. Although metronidazole resistance (7.3%) is relatively low, comparing with other reports, it is emerging in Scotland and may be responsible for treatment failure.

**Figure 3.4 Prevalence of *H. pylori* resistance to 6 antibiotics**



# Chapter 4 Results: Susceptibility Testing for Metronidazole

## 4.1 Introduction

Metronidazole is one of the antibiotics frequently employed in the treatment of *Helicobacter pylori* infection; however, the prevalence rate of metronidazole resistance is variable, ranging from 11 to 70% in developed countries and even more prevalent in developing countries. It is possible that some of the variation in reported resistance levels derives from difficulties in the method of sensitivity testing. Smith and Edwards (1995) indicated that *H. pylori* NCTC 11637 is a metronidazole-resistant strain because it is resistant in microaerophilic conditions and that anaerobic conditions render this strain sensitive. This does raise an important point, as to whether the strains which are metronidazole-resistant *in vitro* in microaerophilic conditions are really true resistant strains *in vivo*. Consequently, the methodology for the determination of susceptibility of *H. pylori* to metronidazole is crucial in the prediction of the success for eradication this bacterium. An evaluation of the variation in methodology on the outcome of susceptibility testing was consequently performed.

## 4.2 The Effect of Pre-incubation under Anaerobic Conditions

To verify the effect of 24 hour pre-incubation in an anaerobic atmosphere, the metronidazole MICs of 21 *H. pylori* strains were determined by E-test. The plates were incubated for 72 hours under microaerophilic conditions, with or without a 24-hour period of anaerobic pre-incubation. *H. pylori* NCTC 11637 was included as a control. The MICs of individual strains are shown in Table 4.1. The comparison of the metronidazole MIC of individual strains determined with and without a 24-hour anaerobic pre-incubation is shown in Figure 4.1.

The E-test had shown that *H. pylori* NCTC 11637 was highly metronidazole-resistant (MIC >256 mg/L) when incubated under microaerophilic conditions. Interestingly, *H. pylori* NCTC 11637 became sensitive to metronidazole with an MIC of 0.016 mg/L when pre-incubated under anaerobic conditions for 24 hours. This suggests that microaerophilic conditions cannot activate metronidazole to its active form. In anaerobic conditions, metronidazole is activated by the reduction of a nitro group to a radical anion which damages the DNA. The requirement for an anaerobic environment for the effective activation of metronidazole may explain why some centres have reported high levels of metronidazole resistance.

A similar finding was observed in 21 *H. pylori* strains. The $MIC_{50}$ and $MIC_{90}$ were 0.5 and >256mg/L, respectively when tested under microaerophilic conditions (Table 4.2 and Figure 4.2). When pre-incubated in anaerobic environment the $MIC_{50}$ and $MIC_{90}$ of

metronidazole decreased to 0.016 and 0.19mg/L for, respectively. The ratio between metronidazole MIC determined with and without pre-incubation under anaerobic conditions is shown in Figure 4.2. All strains, except HP-91, had 2.6 to 16,000-fold decrease in the MIC when pre-incubated under anaerobic conditions. Five strains including the control strain NCTC 11637 had a 16,000-fold decrease in the MIC. A 750-fold decrease in the MIC was observed in one isolate. MIC decrease of 11.8 to 93.7-fold was observed in 9 strains when pre-incubated in anaerobic atmosphere. Only six strains showed less than 10-fold decrease in the MIC.

MICs of most of *H. pylori* strains were decreased significantly when pre-incubated under anaerobic atmosphere. Anaerobic pre-incubation had great effect in the MIC value. Of the 21 *H. pylori* strains, 6 strains were reported as sensitive strains when the MIC was tested under microaerophilic condition, whereas only one resistant strain (HP-91, MIC >256mg/L) had no change in the MIC and might be considered truly resistant. These results explain the variation in the resistance levels reported and suggest that anaerobic pre-incubation should be used to perform the susceptibility testing as metronidazole is still effective in the eradication treatment which is in contrast to a very high prevalence of metronidazole resistance reported.

**Table 4.1 Metronidazole MIC determined with and without pre-incubation under anaerobic conditions**

| Strain | MIC (mg/L) | |
|---|---|---|
| | **Pre-incubation under anaerobic conditions** | **No pre-incubation under anaerobic conditions** |
| HP-18 | 0.016 | 0.094 |
| HP-24 | <0.016 | 0.125 |
| JS139 | <0.016 | 0.125 |
| HP-13 | 0.016 | 0.25 |
| HP-7 | <0.016 | 0.38 |
| HP-17 | 0.016 | 0.38 |
| HP-30 | <0.016 | 0.38 |
| JS133 | 0.016 | 0.38 |
| HP-66 | 0.032 | 0.38 |
| HP-6 | 0.016 | 0.5 |
| HP-61 | <0.016 | 0.5 |
| HP-31 | 0.064 | 0.5 |
| HP-KS | 0.19 | 0.5 |
| HP-PN | 0.19 | 0.5 |
| HP-4 | 0.016 | 1.5 |
| HP-2 | 0.016 | 12 |
| HP-10 | <0.016 | >256 |
| HP-63 | <0.016 | >256 |
| 06640 | 0.016 | >256 |
| *H. pylori* NCTC11916 | 0.016 | >256 |
| HP-91 | >256 | >256 |
| *H. pylori* NCTC11637 | 0.016 | >256 |

**Figure 4.1 Comparison of the metronidazole MIC of individual strains determined with and without pre-incubation under anaerobic conditions**

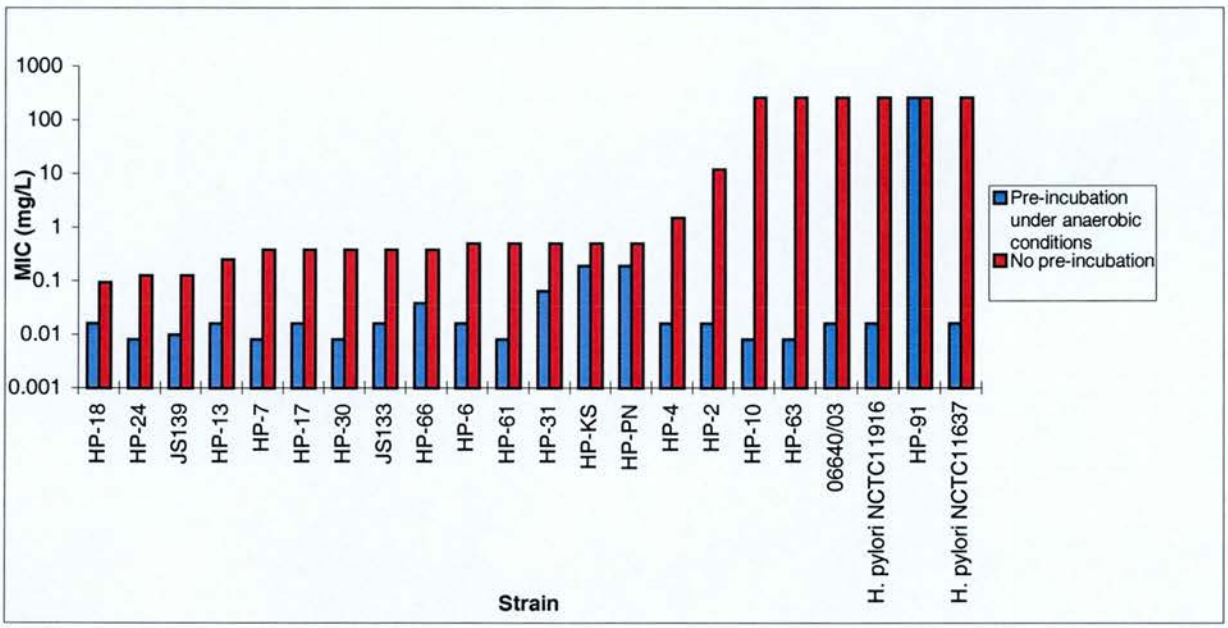



**Table 4.2 $MIC_{50}$ and $MIC_{90}$ of *H. pylori* strains determined with and without a 24-hour anaerobic pre-incubation**

| Incubation conditions | $MIC_{50}$ (mg/L) | $MIC_{90}$ (mg/L) |
|---|---|---|
| Anaerobic pre-incubation | 0.016 | 0.19 |
| No anerobic pre-incubation | 0.5 | >256 |

**Figure 4.2 Comparison of $MIC_{50}$ and $MIC_{90}$ of *H. pylori* strains determined with and without a 24-hour anaerobic pre-incubation**

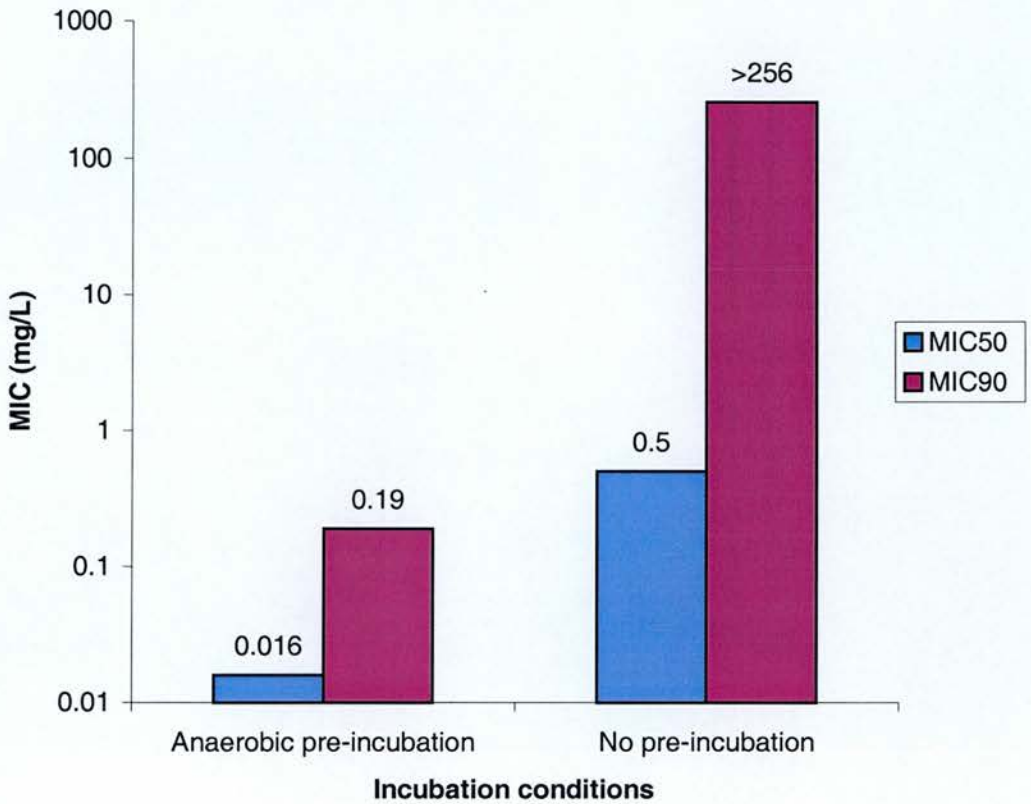
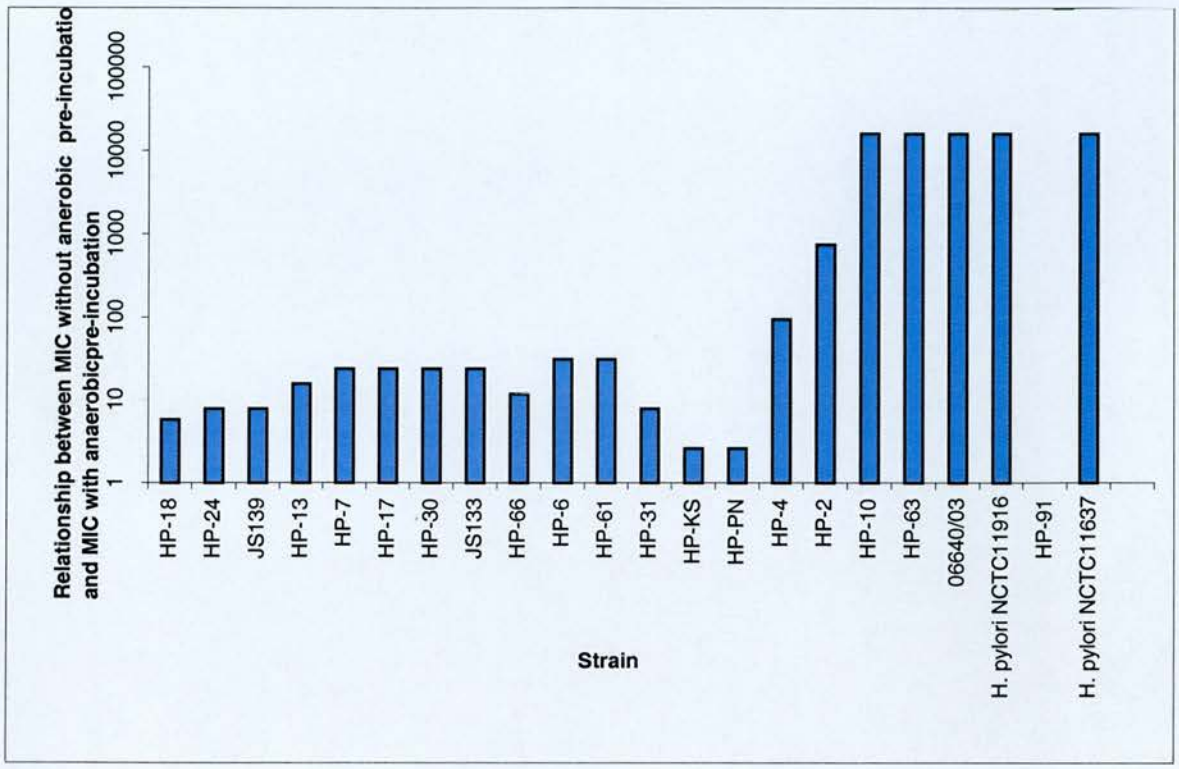


**Figure 4.3 Ratio between MIC determined with and without pre-incubation under anaerobic conditions.**




## 4.3 The Effects of Anaerobic Pre-incubation, Inoculum Size, Medium and Incubation Time

The effect of varying the conditions of susceptibility testing was evaluated with the *H. pylori* NCTC 11637 and NCTC 11916. Table 4.3 shows a comparison of incubation of the MIC plates in an anaerobic jar and an anaerobic chamber (10% $CO_2$, 10% $H_2$ and 80%$N_2$) before transferring to microaerophilic conditions. Inconsistency in *H. pylori* growth was observed when the MIC plates were pre-incubated in an anaerobic chamber.

*H. pylori* grew very well when an anaerobic jar was used to achieve anaerobic conditions. This indicates that *H. pylori* growth depends on a high level of humidity which is not provided in an aerobic chamber. The effects of medium, inoculum size and incubation period were shown in Table 4.4. Columbia and Mueller-Hinton agar were investigated. *H. pylori* growth was poor on Mueller-Hinton agar with 7% horse blood; however, on Columbia agar with 7% horse blood there was significant growth. It was difficult to read the MICs when the inoculum size was 0.5 McFarland because of the poor growth; however, the turbidity of McFarland No.3 provided good growth and reproducibility. After 48-hour incubation under microaerophilic conditions, it was not possible to read the MICs because the growth was too weak. When the plates were incubated for 72 hours, the MICs were easier to read and the results were unequivocal.

The optimal conditions for susceptibility to metronidazole obtained from this study were pre-incubation under anaerobic conditions for 24 hours prior to a 72-hour microaerophilic condition incubation in anaerobic jar on Columbia agar with inoculum size of No.3 McFarland standard. Anaerobic pre-incubation is important to achieve the accurate results.

**Table 4.3 Comparison of anaerobic pre-incubation conditions.** ± indicates poor growth and ++ indicates good growth.

| Medium | Strain | Anaerobic jar | Anaerobic chamber |
|---|---|---|---|
| **Columbia agar** | *H. pylori* NCTC 11916 | ++ | ± |
| | *H. pylori* NCTC 11637 | ++ | ± |

**Table 4.4 Effects of medium (a), inoculum size (b) and incubation time (c).**

± indicates poor growth and ++ indicates good growth.

**(a)**

| Inoculum size | Strain | Medium | |
|---|---|---|---|
| | | Mueller-Hinton agar | Columbia agar |
| **No.3 McFarland** | *H. pylori* NCTC 11916 | ± | ++ |
| | *H. pylori* NCTC 11637 | ± | ++ |

**(b)**

| Medium | Strain | Inoculum size | |
|---|---|---|---|
| | | 0.5 McFarland | No.3 McFarland |
| Columbia agar | *H. pylori* NCTC 11916 | ± | ++ |
| | *H. pylori* NCTC 11637 | ± | ++ |

**(c)**

| Strain | Incubation time | |
|---|---|---|
| | 48 hours | 72 hours |
| *H. pylori* NCTC 11916 | ± | ++ |
| *H. pylori* NCTC 11637 | ± | ++ |

# Chapter 5 Results: Macrolide Resistance

## 5.1 Introduction

Clarithromycin is a potent macrolide that has frequently been used in eradication regimens for *H. pylori* infection. Resistance to clarithromycin varies with geographic location, but it is generally less than 10%. This study shows that the prevalence of clarithromycin resistance in Scotland is 8.2% (See Chapter 3). The development of clarithromycin resistance is a major cause of treatment failure. Macrolide resistance in *H. pylori* is associated with single nucleotide mutation in the peptidyl transferase region of the 23S rRNA. The A to G mutations at either position 2142 or 2143, equivalent to position 2058 or position 2059 in *E. coli*, were found in most isolates associated with clarithromycin resistance.

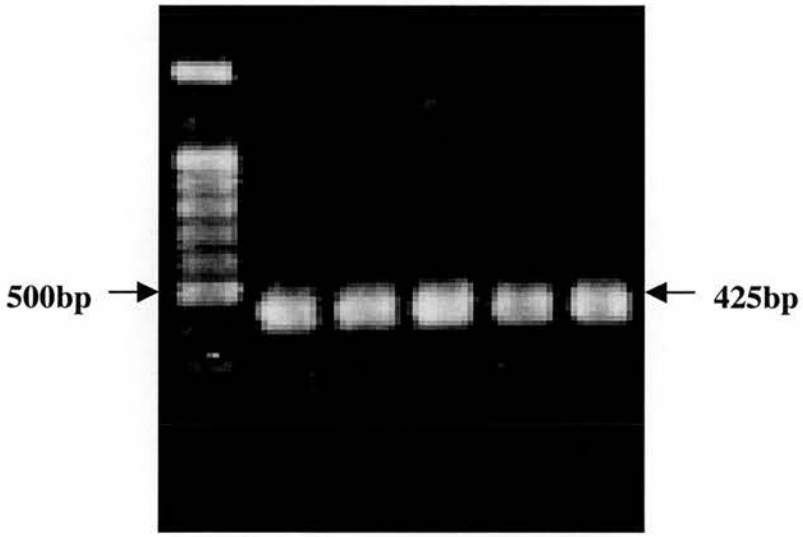
In this study, the 23S rRNA of clinical strains isolated in Scotland was analysed to determine the presense of mutation conferring macrolide resistance.

## 5.2 PCR Amplification of 23S rRNA

Ten macrolide-resistant strains isolated in Scotland, TC-18, TC-19, TC-24, TC-40, TC-78, TC-140, TC-175, TC-185, TC-215 and TC-284, were used in this study. Macrolide resistance conferring mutations in the peptidyl transferase region of the 23S rRNA gene

were determined by PCR amplification and DNA sequencing. A 425bp fragment was amplified by PCR and then sequenced. The results are shown in Figure 7.1. Bands of expected size, 425bp, were observed in all isolates.

**Figure 7.1 PCR amplification of a 425bp fragment of the peptidyl tranferase region of the 23S rRNA**




**Lane 1: 100-bp ladder**

**Lane 2-6: 23S rRNA gene PCR products from TC-18, TC-19, TC-24, TC-40, TC-78**

## 5.3 Mutations in the 23S rRNA

Mutations in the peptidyl transferase region of the 23S rRNA gene were analysed by DNA sequencing. Of the 10 macrolide-resistant isolates, nine isolates were resistant to clarithromycin and cross-resistant to erythromycin. The MICs of erythromycin and clarithromycin are shown Table 5.1. Only one isolate was resistant to erythromycin alone (TC-175, MIC >256mg/L). Nine clarithromycin-resistant strains, including one strain with intermediate resistant (TC-19), had the MIC range of 1.5->256mg/L). All isolates had very high erythromycin MIC of >256mg/L.

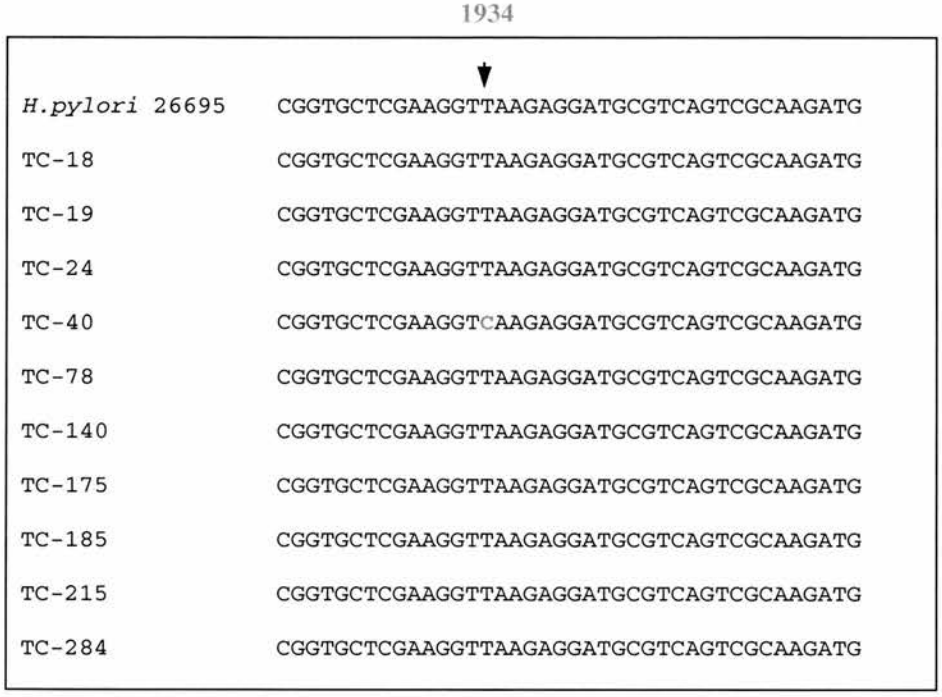
**Table 5.1 Mutations in the 23S rRNA gene of 10 macrolide-resistant *H. pylori***

| Strain | MIC (mg/L) | | 23S rRNA mutations |
|---|---|---|---|
| | Clarithromycin | Erythromycin | |
| TC-18 | >256 | >256 | - |
| TC-24 | >256 | >256 | A2143G, T2182C |
| TC-40 | 32 | >256 | A2143G, T1934C |
| TC-78 | 6 | >256 | A2143G |
| TC-19 | 1.5 | >256 | A2143G |
| TC-140 | 3 | >256 | A2143G |
| TC-175 | 0.125 | >256 | T2182C |
| TC-185 | 24 | >256 | A2143G |
| TC-215 | 3 | >256 | A2143G |
| TC-284 | 8 | >256 | C2195T |

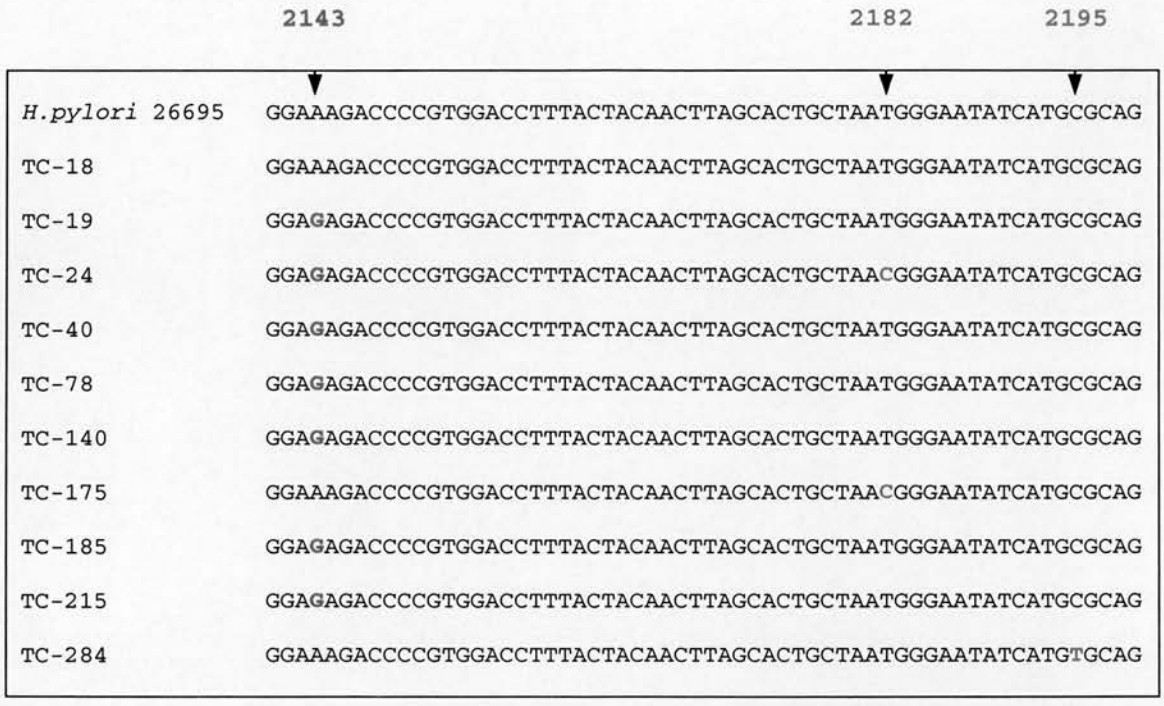
Table 5.1 summarises the mutations that occurred in the 23S rRNA of 10 macrolide-resistant isolates. The alignment of 23S rRNA sequence is shown in Figure 5.2. Sequences of macrolide-resistant isolates were also compared against the sequence of *H. pylori* 26695 from the GenBank database. Sequence analysis revealed four different point mutations in the 23S rRNA (position 2143, 2182, 1934 and 2195) (Table 5.1). The majority of macrolide-resistant isolates contained A to G mutation at position 2143. Seven of the ten macrolide-resistant isolates had mutations at position 2143 (A to G). Two of the seven isolates carried an additional T to C mutation at either position 2182 or 1934. Of the 10 macrolide-resistant isolates, two had a single mutation at either position 2182 (TC-175, erythromycin MIC >256mg/L and clarithromycin MIC= 0.125mg/L) or 2195 (TC-284, erythromycin MIC >256mg/L and clarithromycin MIC =8mg/L). Mutation at position 2182, however, has previously been determined not to be associated with macrolide resistance, indicating that other mechanisms of resistance play a role in TC-175. Mutation at position 2195 may be involved in the mechanism of macrolide resistance, but further investigation is needed to confirm as the results for *H. pylori* strains TC-18 and TC-175 suggest that other additional mechanisms of macrolide resistance exist in *H. pylori*. The mutations at position at 1934 (T to C) and position 2195 (C to T) have not previously been reported. Since T1934C mutation (TC-40) was found together with an A2143G mutation, which is known to confer macrolide resistance in *H. pylori*, the contribution of the T1934C is still unclear. One of the 10 isolates (TC-18, erythromycin and clarithromycin MICs >256 mg/L) had no alteration in the 23S rRNA, suggesting that other mechanisms are involved in macrolide resistance in this strain.

**Figure 5.2 Multiple suquence alignment of the 23S rRNA gene.** (a) mutation at position 1934; (b) mutations at position 2143, 2182 and 2195. Point mutations are indicated in purple, red, blue and pink.

**(a) Mutation at position 1934**



**(b) Mutations at position 2143, 2182 and 2195**



# Chapter 6 Results: Fluoroquinolone Resistance

## 6.1 Introduction

Fluoroquinolones are known to target DNA gyrase. In gram-negative bacteria, DNA gyrase is the primary target of fluoroquinolones. Mutations in the QRDR of the *gyrA* are associated with an increase in MIC of quinolone drugs. In *E. coli*, mutations in serine-83 and aspartate-87 confer high-level of resistance. In *H. pylori*, resistance to fluoroquinolone was found in a clinical trial that examined the efficacy of ciprofloxacin in the treatment of *H. pylori* infection (Moore *et al.* 1995). Sequencing analysis showed that mutations in the *gyrA* gene, encoding DNA gyrase subunit A, at amino acid 87, 88, 91 and double amino acid substitution at positions 91 and 97 were associated with an increase in resistance (MIC). However, *gyrA* mutations yielded ciprofloxacin resistance of 4-8mg/L, which is not very high.

To investigate the development of resistance and identify the target of ciprofloxacin in *H. pylori*, the sequential progression to high-level fluoroquinolone resistance *in vitro* was determined by a stepwise mutation experiment. Mutants of *H. pylori* strains were selected in 3 steps on plates with increasing ciprofloxacin concentrations. To characterise the genetic mutation associated with resistance, the QRDR of the *gyrA* gene of the laboratory mutants was amplified and sequenced. In order to correlate *gyrA* mutations in laboratory mutants and clinical isolates, the *gyrA* gene of *H. pylori* clinical

isolates that exhibited decreased susceptibility to ciprofloxacin were characterised by PCR amplification and DNA sequencing.

## 6.2 In-vitro Fluoroquinolone Selection

To investigate the development of fluoroquinolone resistance in *H. pylori in vitro*, sequential mutations in the QRDR of the *gyrA* gene of five different laboratory ciprofloxacin-resistant mutants were characterised. The mutants were generated from five ciprofloxacin-sensitive strains: HP-1, HP-4, HP-7, HP-17, *H. pylori* NCTC11916.

### 6.2.1 Ciprofloxacin Selection of HP-1 and HP-4

Ciprofloxacin-resistant mutants were selected from *H. pylori* HP-1 and HP-4 in stepwise manner on increasing concentrations of ciprofloxacin. To characterise the order of mutations in the development of ciprofloxacin resistance, the QRDR of the *gyrA* gene (238bp) of all the laboratory mutants and parent strains was amplified by PCR and the fragment was analysed by automated DNA sequencing. The MICs and sequential mutations in *gyrA* are shown in Table 6.1, 6.2 and 6.3. The MICs of the mutants showed decreased sensitivity to ciprofloxacin, with the MIC between 125 and 2000-fold higher than the parent strain HP-1 and 1000 to 16000 fold higher than the parent strain HP-4. The first-step mutants HP-1.1 had the MIC of 8 mg/L, which was a 125-fold increase in ciprofloxacin MIC in comparison to the parent strain (HP-1, MIC = 0.064mg/L). HP-1 showed an amino-acid change in *gyrA* from Aspartic acid-91 to Asparagine. The first-

step mutant from HP–4 (HP-4.1, MIC 16mg/L) also had *gyrA* mutation at position 91 but with amino acid change from Aspartic acid to Tyrosine. The MIC of the first-step mutant increased by 1000-fold compared to the parent strain HP-1 (MIC = 0.016mg/L). The second step mutants HP-1.2 and HP–4.2 (MIC = 64 mg/L) with 8-fold and 4-fold increase in the MIC, respectively, had no change in the sequences. No alterations in the QRDR of the *gyrA* gene were observed in the third-step mutants (MIC = 128mg/L for HP–1.3 and MIC = 256mg/L for HP-4.3) with 2-fold and 4-fold increase in the MIC.

Mutations at Aspartic acid-91 to either Asparagine or Tyrosine are associated with high-level fluoroquinolone resistance in *H. pylori*. No additional mutation was found in the second-step and third-step mutations of HP–1 and HP–4, suggesting that other mechanisms of resistance may play a role in the mechanism of ciprofloxacin resistance in *H. pylori*.

**Table 6.1 Mutation in the QRDR of ciprofloxacin-resistant mutants from HP-1 and HP-4**

| Strain | MIC (mg/L) | *gyrA* mutations |
|---|---|---|
| **Parent** | | |
| HP-1 | 0.064 | - |
| HP-4 | 0.016 | - |
| **1st Step** | | |
| HP-1.1 | 8 | Asp-91 → Asn |
| HP-4.1 | 16 | Asp-91 → Tyr |
| **2nd Step** | | |
| HP-1.2 | 64 | Asp-91 → Asn |
| HP-4.2 | 64 | Asp-91 → Tyr |
| **3rd Step** | | |
| HP-1.3 | 128 | Asp-91 → Asn |
| HP-4.3 | 256 | Asp-91 → Tyr |

**Table 6.2 Comparison of partial DNA sequences of ciprofloxacin-resistant mutants of HP-1.** The deduced amino acid sequences are given below the nucleotide sequences. Letters in red indicate point mutations, leading to amino-acid substitutions.

| Strain | Mutations in the QRDR of *gyrA* | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Codon** | **80** | **81** | **82** | **83** | **84** | **85** | **86** | **87** | **88** | **89** | **90** | **91** | **92** | **93** |
| HP-1 | | aaa | tac | cac | ccc | cat | ggc | gat | aat | gcg | gtt | tat | gat | gcg | cta |
| | | K | Y | H | P | H | G | D | N | A | V | Y | D | A | L |
| HP-1.1 | | aaa | tac | cac | ccc | cat | ggc | gat | aat | gcg | gtt | tat | aat | gcg | cta |
| | | K | Y | H | P | H | G | D | N | A | V | Y | N | A | L |
| HP-1.2 | | aaa | tac | cac | ccc | cat | ggc | gat | aat | gcg | gtt | tat | aat | gcg | cta |
| | | K | Y | H | P | H | G | D | N | A | V | Y | N | A | L |
| HP-1.3 | | aaa | tac | cac | ccc | cat | ggc | gat | aat | gcg | gtt | tat | aat | gcg | cta |
| | | K | Y | H | P | H | G | D | N | A | V | Y | N | A | L |

**Table 6.3 Comparison of partial DNA sequences of ciprofloxacin-resistant mutants of HP-4.** The deduced amino acid sequences are given below the nucleotide sequences. Letters in red indicate point mutations, leading to amino acid substitutions.

| Strain | Mutations in the QRDR of *gyrA* | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Codon** | **80** | **81** | **82** | **83** | **84** | **85** | **86** | **87** | **88** | **89** | **90** | **91** | **92** | **93** |
| HP-4 | | aaa | tac | cac | ccc | cat | ggc | gat | aac | gcg | gtt | tat | gat | gcg | cta |
| | | K | Y | H | P | H | G | D | N | A | V | Y | D | A | L |
| HP-4.1 | | aaa | tac | cac | ccc | cat | ggc | gat | aac | gcg | gtt | tat | tat | gcg | cta |
| | | K | Y | H | P | H | G | D | N | A | V | Y | Y | A | L |
| HP-4.2 | | aaa | tac | cac | ccc | cat | ggc | gat | aac | gcg | gtt | tat | tat | gcg | cta |
| | | K | Y | H | P | H | G | D | N | A | V | Y | Y | A | L |
| HP-4.3 | | aaa | tac | cac | ccc | cat | ggc | gat | aac | gcg | gtt | tat | tat | gcg | cta |
| | | K | Y | H | P | H | G | D | N | A | V | Y | Y | A | L |

### 6.2.2 Ciprofloxacin Selection of HP-7 and *H. pylori* NCTC11916

The MICs and sequential mutations in *gyrA* of laboratory mutants of HP–7 and *H. pylori* NCTC11916 are shown in Table 6.4, 6.5 and 6.6. The mutants showed decreased sensitivity to ciprofloxacin, with the MICs between 500- and 16000-fold higher than the parent strain HP-7 and 250- to 2000-fold higher than the parent strain *H. pylori* NCTC 11916. The two first-step mutants, with the MIC of 16mg/L (HP7.1 and NCTC 11916A), had the same *gyrA* mutation at Aspartic acid 91 to Asparagine and there was 500-fold and 250-fold increase in ciprofloxacin MIC in comparison to the parent strain (HP-7, MIC = 0.032mg/L, NCTC11916, MIC = 0.064mg/L). The second-step mutant HP-7.2 had the MIC of 128 mg/L with a 8-fold increase in the MIC and carried an additional mutation at Asparagine-87 to Lysine. The second-step mutation of NCTC 11916 (NCTC11916B) raised the MIC to 64 mg/L, with MIC increasing by 4 fold, but no change was found in the sequence. On the other hand, the third step mutation of NCTC 11916 (NCTC11916C, MIC 128 mg/L) had the additional mutation at Asparagine-87 to Lysine, with a two-fold increase in the MIC. The third-step mutation of HP-7 (HP-7.3, MIC 512 mg/L) had no amino-acid changes in sequence and the MIC increased by 4-fold. Silent nucleotide substitutions, which did not confer amino-acid changes, were found at positions 87 and 93 in the first-step mutant HP-7.1 and at positions 73, 78, 82 and 88 in the second- and third-step mutant of HP-7. The third-step mutant of *H. pylori* NCTC11916 had silent mutations at positions 73, 78, 82 and 88.

A single amino-acid substitution at Aspartic acid-91 to Asparagine or a double amino substitutions at Aspartic acid-91 to Asparagine or Asparagine-87 to Lysine are

associated with fluoroquinolone resistance in *H. pylori*. High-level resistance to ciprofloxacin was associated with two mutation sites in the *gyrA* gene in laboratory mutants. Other mechanisms of resistance, besides target site mutations, may play a role in the mechanisms of ciprofloxacin resistance in *H. pylori*, as no additional mutation was identified in the second-step mutation of NCTC11916 and the third-step mutation of HP-7.

**Table 6.4 Mutation in the QRDR of ciprofloxacin-resistant mutants from HP-7 and *H. pylori* NCTC11916**

| Strain | MIC (mg/L) | *gyrA* mutations |
|---|---|---|
| **Parent** | | |
| HP-7 | 0.032 | - |
| NCTC11916 | 0.064 | - |
| **1st Step** | | |
| HP-7.1 | 16 | Asp-91 → Asn |
| NCTC11916A | 16 | Asp-91 → Asn |
| **2nd Step** | | |
| HP-7.2 | 128 | Asp-91 → Asn, Asn-87 → Lys |
| NCTC11916B | 64 | Asp-91 → Asn |
| **3rd Step** | | |
| HP-7.3 | 512 | Asp-91 → Asn, Asn-87 → Lys |
| NCTC11916C | 128 | Asp-91 → Asn, Asn-87 → Lys |

**Table 6.5 Comparison of partial DNA sequences of ciprofloxacin-resistant mutants of HP-7.** The deduced amino-acid sequences are given below the nucleotide sequences. Letters in red indicate point mutations, leading to amino-acid substitutions. Letters in green indicate silent nucleotide mutations.

| Strain | Mutations in the QRDR of *gyrA* | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Codon** | **80** | **81** | **82** | **83** | **84** | **85** | **86** | **87** | **88** | **89** | **90** | **91** | **92** | **93** |
| HP-7 | | aaa | tac | cac | ccc | cat | ggc | gat | aat | gcg | gtt | tat | gat | gcg | tta |
| | | K | Y | H | P | H | G | D | N | A | V | Y | D | A | L |
| HP-7.1 | | aaa | tac | cac | ccc | cat | ggc | gat | aac | gcg | gtt | tat | aat | gcg | cta |
| | | K | Y | H | P | H | G | D | N | A | V | Y | N | A | L |
| HP-7.2 | | aaa | tac | cat | ccc | cat | ggc | gat | aaa | gca | gtt | tat | aat | gcg | cta |
| | | K | Y | H | P | H | G | D | K | A | V | Y | N | A | L |
| HP-7.3 | | aaa | tac | cat | ccc | cat | ggc | gat | aaa | gca | gtt | tat | aat | gcg | cta |
| | | K | Y | H | P | H | G | D | K | A | V | Y | N | A | L |

**Table 6.6 Comparison of partial DNA sequences of ciprofloxacin-resistant mutants of *H. pylori* NCTC11916.** The deduced amino-acid sequences are given below the nucleotide sequences. Letters in red indicate point mutations, leading to amino-acid substitutions. Letters in green indicate silent nucleotide mutations.

| Strain | Mutations in the QRDR of *gyrA* | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Codon** | **80** | **81** | **82** | **83** | **84** | **85** | **86** | **87** | **88** | **89** | **90** | **91** | **92** | **93** |
| NCTC11916 | | aaa | tac | cac | ccc | cat | ggc | gat | aat | gcg | gtt | tat | gat | gcg | cta |
| | | K | Y | H | P | H | G | D | N | A | V | Y | D | A | L |
| NCTC11916A | | aaa | tac | cac | ccc | cat | ggc | gat | aat | gcg | gtt | tat | aat | gcg | cta |
| | | K | Y | H | P | H | G | D | N | A | V | Y | N | A | L |
| NCTC11916B | | aaa | tac | cac | ccc | cat | ggc | gat | aat | gcg | gtt | tat | aat | gcg | cta |
| | | K | Y | H | P | H | G | D | N | A | V | Y | N | A | L |
| NCTC11916C | | aaa | tac | cat | ccc | cat | ggc | gat | aaa | gca | gtt | tat | aat | gcg | cta |
| | | K | Y | H | P | H | G | D | K | A | V | Y | N | A | L |

### 6.2.3 Ciprofloxacin Selection of HP-17

The MICs and sequential mutations in *gyrA* of the laboratory mutants of HP-17 are shown in Table 6.7 and 6.8. The mutants showed decreased sensitivity to ciprofloxacin, with the MIC between 250- and 4000-fold higher than the parent strain HP-17. The first-step mutant had an MIC of 8mg/L with a 250-fold increase in the MIC compared to the parent strain HP-17 (MIC = 0.032mg/L). Sequence analysis showed a mutation in the *gyrA* gene at Aspartic acid-91 to Asparagine. The second-step mutant (MIC 64mg/L) carried an additional mutation at Glycine-85 to Cysteine with ciprofloxacin MIC increasing by 8 fold. Interestingly, the third-step mutant had a *gyrA* change at amino acid 91 again, changing from Asparagine to Tyrosine and associated with ciprofloxacin MIC of 128mg/L, which is a 2-fold increase in the MIC.

A single amino-acid substitution at Aspartic acid-91 to Asparagine or a double amino substitution at Aspartic acid-91 to either Asparagine or Tyrosine, and Glycine-85 to Cysteine confer fluoroquinolone resistance in *H. pylori*. It was demonstrated that high-level ciprofloxacin was associated with a double amino-acid change in the *gyrA* gene the laboratory mutants.

**Table 6.7 Mutations in the QRDR of *gyrA* in ciprofloxacin-resistant mutants of HP-17.**

| Strain | MIC (mg/L) | *gyrA* mutations |
|---|---|---|
| **Parent** | | |
| HP-17 | 0.032 | - |
| **1st Step** | | |
| HP-17.1 | 8 | Asp-91 → Asn |
| **2nd Step** | | |
| HP-17.2 | 64 | Asp-91 → Asn, Gly-85 → Cys |
| **3rd Step** | | |
| HP-17.3 | 128 | Asp-91 → Tyr, Gly-85 → Cys |

**Table 6.8 Comparison of partial DNA sequences of ciprofloxacin-resistant mutants of HP-17.** The deduced amino acid sequences are given below the nucleotide sequences. Letters in red indicate point mutations, leading to amino acid substitutions.

| Strain | Mutations in the QRDR of *gyrA* | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Codon** | **80** | **81** | **82** | **83** | **84** | **85** | **86** | **87** | **88** | **89** | **90** | **91** | **92** | **93** |
| HP-17 | | aaa | tac | cac | ccc | cat | ggc | gat | aat | gcg | gtt | tat | gat | gcg | tta |
| | | K | Y | H | P | H | G | D | N | A | V | Y | D | A | L |
| HP-17.1 | | aaa | tac | cac | ccc | cat | ggc | gat | aat | gcg | gtt | tat | aat | gcg | tta |
| | | K | Y | H | P | H | G | D | N | A | V | Y | N | A | L |
| HP-17.2 | | aaa | tac | cac | ccc | cat | tgc | gat | aat | gcg | gtt | tat | aat | gcg | tta |
| | | K | Y | H | P | H | C | D | N | A | V | Y | N | A | L |
| HP-17.3 | | aaa | tac | cac | ccc | cat | tgc | gat | aat | gcg | gtt | tat | tat | gcg | tta |
| | | K | Y | H | P | H | C | D | N | A | V | Y | Y | A | L |

## 6.3 Clinical Fluoroquinolone Resistance

In the previous section (Section 6.2), I have identified the mechanism of fluoroquinolone resistance by target site mutations in that occurred *in vitro*. This study further examined the contribution of the *gyrA* gene to the development of resistance to fluoroquinolone in *H. pylori in vivo*. Mutations in the *gyrA* gene that confer fluoroquinolone resistance in three ciprofloxacin-resistant *H. pylori* clinical isolates were characterised. These isolates were isolated in Scotland. TC-246 (MIC = 16mg/L) and TC-413 (MIC = >32mg/L) were the isolates obtained from this project (Chapter 3). TC-24 (MIC =32mg/L) was obtained from the Infection and Immunity laboratory, University of Edinburgh.

The QRDR of the *gyrA* gene from each isolate was amplified by PCR. A 238bp fragment was analysed by automated DNA sequencing and compared with the ciprofloxacin-sensitive *H. pylori* NCTC 11916. Analysis of the QRDR of the *gyrA* gene showed that all three ciprofloxacin-resistant isolates harboured a *gyrA* mutation (Table 6.9).

**Table 6.9 Amino acid substitutions in the QRDR of *gyrA* in clinical isolates**

| Strain | MIC (mg/L) | *gyrA* mutation |
|---|---|---|
| TC-246 | 16 | Asp-91→ Asn |
| HP-24 | 32 | Asn-87 → Lys |
| TC-413 | >32 | Asp-91→ Asn |

Sequence analysis revealed that TC-246 and TC-413 had a GAT-to-AAT mutation, in codon 91, changing from Aspartic acid to Asparagine. HP-24 carried a single amino acid substitution at Asparagine-87 to Lysine (AAT to AAA) and silent nucleotide substitutions, which did not confer amino-acid changes at positions 82 and 88. Table 6.10 shows the sequence alignment and corresponding amino-acid substitution of *gyrA*. Nucleotide sequence of the *gyrA* fragment obtained from the wild-type ciprofloxacin-sensitive *H. pylori* NCTC 11916 was used as a control.

Mutations conferring ciprofloxacin resistance in clinical isolates from this study demonstrated that a single amino-acid substitution in the QRDR of the *gyrA* gene is responsible for fluoroquinolone resistance in *H. pylori in vivo*. Amino-acid substitutions at position 91 (Aspartic acid to Asparagine) and position 87 (Asparagine to Lysine) were found in the mutation studies in the laboratory mutants in this study and were also identified in the clinical isolates from the study by Moore *et al.* (1995).

**Table 6.10 Comparison of partial DNA sequences of ciprofloxacin-resistant clinical isolates.** The deduced amino acid sequences are given below the nucleotide sequences. Letters in red indicate point mutations, leading to amino acid substitutions. Letters in green indicate silent nucleotide mutations.

| Strain | Mutations in the QRDR of *gyrA* | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Codon** | **80** | **81** | **82** | **83** | **84** | **85** | **86** | **87** | **88** | **89** | **90** | **91** | **92** | **93** |
| NCTC 11916 | | Aaa | tac | cac | ccc | cat | ggc | gat | aat | gcg | gtt | tat | gat | gcg | cta |
| | | K | Y | H | P | H | G | D | N | A | V | Y | D | A | L |
| HP-24 | | aaa | tac | cat | ccc | cat | ggc | gat | aaa | gca | gtt | tat | gat | gcg | cta |
| | | K | Y | H | P | H | G | D | K | A | V | Y | D | A | L |
| TC-246 | | aaa | tac | cac | ccc | cat | ggc | gat | aac | gcg | gtt | tat | aat | gcg | cta |
| | | K | Y | H | P | H | G | D | N | A | V | Y | N | A | L |
| TC-413 | | aaa | tac | cac | ccc | cat | ggc | gat | aac | gcg | gtt | tat | aat | gcg | cta |
| | | K | Y | H | P | H | G | D | N | A | V | Y | N | A | L |

# Chapter 7 Results: Metronidazole Resistance

## 7.1 Introduction

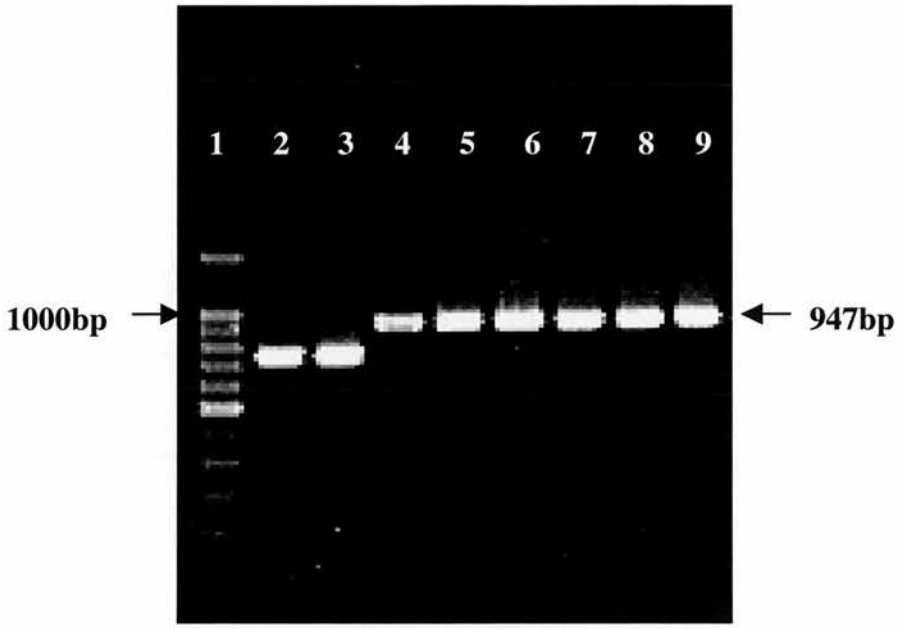
Metronidazole is an important component of many currently used *H. pylori* treatment regimens and resistance to metronidazole is one of the major causes of treatment failure. Metronidazole resistance was 16-42% in Europe (Megraud *et al.*, 1999) and 37% in the US (Osato *et al.*, 1999). The prevalence of metronidazole resistance is far higher in developing countries, where metronidazole is used to treat anaerobic and parasitic infections. Metronidazole resistance rate was 90% in India (Mukhopadhyay *et al.*, 2000) and 78% in China (Wu *et al.*, 2000). Several mechanisms have been proposed for the metronidazole resistance, such as enhanced scavenging of toxic oxygen radicals by an altered catalase or superoxide dismutase and an efficient DNA damage repair mechanism. Goodwin *et al.* (1998) recently reported that the loss of oxygen-insensitive NADPH nitroreductase activity resulted in the development of metronidazole resistance in *H. pylori*. The *rdxA* gene encodes an oxygen-insensitive NADPH nitroreductase that reduces metronidazole to its active metabolite that is toxic for *H. pylori*. It has been proposed that mutational inactivation of *rdxA* confers metronidazole resistance (Goodwin *et al.*, 1998). However, an intact *rdxA* gene has also been reported in metronidazole-resistant *H. pylori* (Jenks *et al.*, 1999). To investigate the contribution of metronidazole to the development of metronidazole resistance, the *rdxA* gene of metronidazole-resistant *H. pylori* isolated from Scotland was amplified and then sequenced. This study also examined the *rdxA* gene of *H. pylori* NCTC 11637 that has

been reported as a metronidazole-resistant strain but when tested for metronidazole resistance by our susceptibility method (a 24-hour anaerobic pre-incubation), it behaves to be a sensitive strain.

## *7.2* PCR Amplification of *rdxA*

The *rdxA* gene encoding oxygen-insensitive NADPH nitroreductase was amplified by PCR from six metronidazole-resistant strains isolated in Scotland, TC-18, TC-165, TC-232, TC-243, TC-296 and TC-376 (MIC range, 4 - >256mg/L) and two sensitive strains, *H. pylori* NCTC11637 and NCTC11916 (MIC = 0.016mg/L), producing a 947bp fragment. The results are shown in Figure 7.1.

**Figure 7.1 Confirmation *rdxA* fragment sizes**




**Lane 1: 100-bp ladder**

**Lane 2-9: *rdxA* PCR products from *H. pylori* NCTC11637, NCTC11916, TC-18, TC-165, TC-232, TC-243, TC-296 and TC-376.**

Bands of expected size, 947bp, were observed in all resistant strains but not for the two sensitive strains, NCTC11637 and NCTC11916. PCR products from NCTC11637 and NCTC11916 yielded a product of approximately 200bp smaller than the expected size and those of other resistant strains, suggesting that deletions have occurred in the sequences. All PCR products were then further characterised by DNA sequencing.

## 7.3 Mutations in *rdxA*

Mutations in each of the *rdxA* gene were analysed by DNA sequencing. The PCR products were purified by Qiaquick PCR purification kit and sequenced using the PCR primers. The *rdxA* sequences were compared to *H. pylori* 26695 *rdxA* in GenBank, with accession number AE000604. Amino-acid substitutions and alignment of RdxA of 6 metronidazole-resistant strains is shown in Table 7.1 and Figure 7.2.

**Table 7.1 Amino acid substitutions in RdxA of metronidazole resistant-isolates.** The RdxA sequence of *H. pylori* 26695 (GenBank) was used to compare with the metronidazole-resistant isolates. Dots indicate identical amino acids. # indicates a stop codon.

| Strain | Amino-acid position | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **6** | **16** | **24** | **31** | **51** | **52** | **55** | **59** | **62** | **71** | **75** | **83** | **85** | **90** | **97** | **103** | **114** | **118** | **172** |
| HP26695 | Q | R | S | T | P | W | V | D | L | Y | E | L | V | R | H | N | I | A | V |
| TC-18 | H | C | . | . | . | . | . | N | . | . | . | . | . | K | T | . | . | T | I |
| TC-165 | . | . | . | . | . | . | L | N | . | . | . | V | . | K | . | T | . | . | . |
| TC-232 | . | . | Y | E | . | # | | | | | | | | | | | | | |
| TC-243 | . | . | . | . | . | . | . | N | V | . | K | . | L | . | . | . | L | . | . |
| TC-296 | . | . | . | . | . | . | . | N | . | F | . | . | . | . | . | . | . | S | . |
| TC-376 | . | . | . | E | L | . | . | N | . | | . | . | . | . | . | . | . | S | I |

**Figure 7.2 Alignment of RdxA proteins of 6 metronidazole-resistant isolates.** The RdxA sequences of metronidazole-resistant strains were compared to that of *H. pylori* 26695. Letters in red show amino acids in the conserved region of the RdxA protein (amino acids positions 43 to 57). Letters in red indicate amino-acid substitutions. Stop codon is indicated by #.

```
1                                                          43    50
HP26695  MKFLDQEKRR QLLNERHSCK MFDSHYEFSS TELEEIAEIA RLSPSSYNTQ
TC-296   MKFLDQEKRR QLLNERHSCK MFDSHYEFSS TELEEIAEIA RLSPSSYNTQ
TC-243   MKFLDQEKRR QLLNERHSCK MFDSHYEFSS TELEEIAEIA RLSPSSYNTQ
TC-165   MKFLDQEKRR QLLNERHSCK MFDSHYEFSS TELEEIAEIA RLSPSSYNTQ
TC-376   MKFLDQEKRR QLLNERHSCK MFDSHYEFSS EELEEIAEIA RLSPSSYNTQ
TC-18    MKFLDHEKRR QLLNECHSCK MFDSHYEFSS TELEEIAEIA RLSPSSYNTQ
TC-232   MKFLDQEKRR QLLNERHSCK MFDYHYEFSS EELEEIAEIA RLSPSSYNTQ

         51    57                                             100
HP26695  PWHFVMVTDK DLKKQIAAHS YFNEEMIKSA SALMVVCSLR PSELLPHGHY
TC-296   PWHFVMVTNK DLKKQIAAHS FFNEEMIKSA SALMVVCSLR PSELLPHGHY
TC-243   PWHFVMVTNK DVKKQIAAHS YFNEKMIKSA SALMLVCSLR PSELLPHGHY
TC-165   PWHFLMVTNK DLKKQIAAHS YFNEEMIKSA SAVMVVCSLK PSELLPHGHY
TC-376   LWHFVMVTNK DLKKQIAAHS YFNEEMIKSA SALMVVCSLR PSELLPHGHY
TC-18    PWHFVMVTNK DLKKQIAAHS YFNEEMIKSA SALMVVCSLK PSELLPTGHY
TC-232   P#

         101                                                  150
HP26695  MQNLYPESYK VRVIPSFAQM LGVRFNHSMQ RLESYILEQC YIAVGQICMG
TC-296   MQNLYPESYK VRVIPSFSQM LGVRFNHSMQ RLESYILEQC YIAVGQICMG
TC-243   MQNLYPESYK VRVLPSFAQM LGVRFNHSMQ RLESYILEQC YIAVGQICMG
TC-165   MQTLYPESYK VRVIPSFAQM LGVRFNHSMQ RLESYILEQC YIAVGQICMG
TC-376   MQNLYPESYK VRVIPSFSQM LGVRFNHSMQ RLESYILEQC YIAVGQICMG
TC-18    MQNLYPESYK VRVIPSFTQM LGVRFNHSMQ RLESYILEQC YIAVGQICMG
TC-232

         151                                                  200
HP26695  VSLMGLDSCI IGGFDPLKVG EVLEERINKP KIACLIALGK RVAEASQKSR
TC-296   VSLMGLDSCI IGGFDPLKVG EVLEERINKP KIACLIALGK RVAEASQKSR
TC-243   VSLMGLDSCI IGGFDPLKVG EVLEERINKP KIACLIALGK RVAEASQKSR
TC-165   VSLMGLDSCI IGGFDPLKVG EVLEERINKP KIACLIALGK RVAEASQKSR
TC-376   VSLMGLDSCI IGGFDPLKVG EILEERINKP KIACLIALGK RVAEASQKSR
TC-18    VSLMGLDSCI IGGFDPLKVG EILEERINKP KIACLIALGK RVAEASQKSR
TC-232

         201    210
HP26695  KSKVDAITWL
TC-296   KSKVDAITWL
TC-243   KSKVDAITWL
TC-165   KSKVDAITWL
TC-376   KSKVDAITWL
TC-18    KSKVDAITWL
TC-232
```

There were 20 different amino-acid changes (positions 6, 16, 24, 31, 51, 52, 55, 59, 62, 71, 75, 83, 85, 90, 97, 103, 114, 118 and 172) in *rdxA* of metronidazole-resistant strains, comparing with the *rdxA* gene of *H. pylori* 26695. The *rdxA* gene of all six metronidazole-resistant strains contained missense mutations that resulted in amino-acid substitutions. One isolate, TC-232, had an additional nonsense mutation at position 52, resulting in a stop codon. All strains had two to seven amino-acid changes in the sequences. Five strains contained the same amino-acid substitution at position 59, changing from Aspartic acid (D) to Asparagine (N). Two of the substitutions, Proline (P) to Leucine (L) at position 51 in TC-376 and a Valine (V) to Leucine (L) at position 55 in TC-165, were at position within a region that is highly conserved in classical oxygen-insensitive NADPH nitroreductase (position 43-57). Three isolates had mutations at the same position (position 118) but different in amino-acid substitutions. Two of these three isolates had Alanine (A) to Serine (S) substitutions (TC-296 and TC-376) and one isolate (TC-18) had an Alanine (A) to Threonine (T) substitution. Amino-acid changes from Threonine (T) to Glutamic acid (E) were found in two isolates (TC-232 and TC-376) at position 31. Amino acid changes from Arginine (R) to Lysine (K) at position 90 were found in TC-18 and TC-165. Two isolates had a Valine (L) to Isoleucine (I) substitution at position 172 (TC-18 and TC-376). The other amino-acid changes were Glutamine (Q) to Histidine (H) (position 6), Arginine (R) to Cysteine (C) (position 16), Serine (S) to Tyrosine (Y) (Position 24), Leucine (L) to Valine (V) (position 62), Tyrosine (Y) to Phenylalanine (F) (position 71), Glutamic acid (E) to Lysine (K) (position 75), Leucine (L) to Valine (V) (position 83), Valine (V) to Leucine (L) (Position 85), Histidine (H) to Threonine (T) (position 97), Asparagine (N) to Threonine

(T) (position 103), Isoleucine (I) to Leucine (L) (position 114). There is no correlation between MIC for metronidazole-resistant isolates and either the type or position of mutation in the *rdxA* gene.

The alignment of RdxA protein of metronidazole-sensitve strains, *H. pylori* NCTC 11637 and NCTC11916, comparing them with *H. pylori* 26695, are shown in Figure 7.3. Both *H. pylori* NCTC 11637 and NCTC 11916 had the same amino-acid changes. Four different amino-acid substitutions were found at amino acid position 14 (Asparagine to Lysine), position 31 (Threonine to Glutamic acid), position 36 (Isoleucine to Valine) and position 56 (Methionine to Isoleucine). Amino-acid substitution at position 56 is in the conserved region. Both strains had nonsense mutations at position 73, leading to a stop codon. My result is different from the study of the *rdxA* sequence of *H. pylori* NCTC 11637 by Debets-Ossenkopp *et al* (1999), who found nonsense mutation at position 83.

**Figure 7.3 Alignment of RdxA protein of metronidazole-sensitive strains.** The sequences were compared to the amino acids of *H. pylori* 26695. Letters in green show amino acids in the conserved region of the RdxA protein (amino acids positions 43 to 57). Letters in red indicate amino-acid substitutions. Letters in blue show amino acid in the insertion region. Dash indicates a stop codon.

```
                1                                                 43      50
   HP26695      MKFLDQEKRR QLLNERHSCK MFDSHYEFSS TELEEIAEIA RLSPSSYNTQ
 NCTC11916      MKFLDQEKRR QLLKERHSCK MFDSHYEFSS EELEEVAEIA RLSPSSYNTQ
 NCTC11637      MKFLDQEKRR QLLKERHSCK MFDSHYEFSS EELEEVAEIA RLSPSSYNTQ

                51     57                                           100
   HP26695      PWHFVMVTDK DLKKQIAAHS YFNEEMIKSA SALMVVCSLR PSELLPHGHY
NCTC 11916      PWHFVIVTNK DLNHPSRNPK HL-
NCTC 11637      PWHFVIVTNK DLNHPSRNPK HL-
```

Sequence analysis showed that these two metronidazole-sensitive strains had insertions of a 267-bp fragment and deletions of a 469-bp segment starting at nucleotide position 189. This can explain the smaller size of PCR products of these strains, which are 202bp smaller than the expected size (947bp). The sequence of the 267-bp insertion is shown in Figure 7.4. A BLAST search revealed that the 267-bp insertion fragment is closely matched to the *H. pylori* vacuolating cytotoxic gene and cag I pathogenicity island. The

41 and 33bp at the left and right ends of the insertion (Figure 7.4) are closely matched to the left and right ends of IS*605*, a 2-kb insertion sequence. Censini *et al* (1996) found the is*605*, a variant of IS*605*, at the right end of *cag* pathogenicity island in *H. pylori* strain CCUG 17874. The is*605* is composed of two arms of IS*605* with no association in the ORFs.

**Figure 7.4 Alignment of the mini-IS*605* sequence present in the *rdxA of H. pylori* NCTC11637 and NCTC11916.** Letters in blue indicate a 267bp insertion. Letters in bold indicate the left and right ends of the insertion which correspond to the mini-IS*605* found at the right end of the *cag* pathogenic island.

```
            189
            ▼
NCTC11916   CCACCCAAGC AGAAATCCCA AACATCTTTA GTGTTTGGGA TGAATGCTGC
NCTC11637   CCACCCAAGC AGAAATCCCA AACATCTTTA GTGTTTGGGA TGAATGCTGC

NCTC11916   TAATTTGTAG TATAATATCT CCATACATTT GTATCTAGCG TAGGAAGTAC
NCTC11637   TAATTTGTAG TATAATATCT CCATACATTT GTATCTAGCG TAGGAAGTAC

NCTC11916   GCAAAGTTAC GCCTTTGGAG ATATGATGTG TGAGACCTGT AGGGAATGCG
NCTC11637   GCAAAGTTAC GCCTTTGGAG ATATGATGTG TGAGACCTGT AGGGAATGCG

NCTC11916   TTGGAGATCA AACTCTGTAA AATCCCTATG ATTAGGGACA CAAAGTGAGA
NCTC11637   TTGGAGATCA AACTCTGTAA AATCCCTATG ATTAGGGACA CAAAGTGAGA

NCTC11916   ACCAAACTTT CCCTATGGGC AACATCAGCC GAGGAAGCCC AATCGCTTTA
NCTC11637   ACCAAACTTT CCCTATGGGC AACATCAGCC GAGGAAGCCC AATCGCTTTA

NCTC11916   GCGTTTGGGT GCTTCAC
NCTC11637   GCGTTTGGGT GCTTCAC
```

Table 7.2 summarises mutations in the RdxA protein. The mutations were compared to metronidazole-sensitive *H. pylori* 26695. The majority of changes in the *rdxA* gene were the results of missense mutations, leading to amino-acid substitution. Alterations in *rdxA* were found in both sensitive and resistant strains. Only three strains had mutations causing a premature stop codon, two of which were metronidazole-sensitive strains. Therefore, only one metronidazole-resistant isolate had a truncated Rdx protein. The majority of amino-acid substitutions found in this study were reported in sensitive strains by other published papers. Only two of them (position 24 and 103) have not been yet been identified. The *rdxA* gene of *H. pylori* has been reported to have about 95% similarlity among *H. pylori* strains. The amino-acid substitutions found in resistant strains may not be responsible for the mechanism of resistance. The alterations in the *rdxA* gene of the two sensitive strains from this study generated a truncated protein, suggesting that other mechanisms of metronidazole resistance exist. The results also indicate that mutations in the *rdxA* gene do not always contribute to the mechanism of metronidazole resistance. An alternative mechanism must be involved in the resistance mechanism.

**Table 7.2 Mutations in the *rdxA* gene of 8 *H. pylori* strains**

| Strain | MIC (mg/L) | RdxA mutations |
|---|---|---|
| *H. pylori* NCTC11637 | 0.016 | Insertion of a 267bp fragment and deletion of a 469bp segment, amino aqcid substitutions at positions 14, 31, 36 and 56 |
| *H. pylori* NCTC11916 | 0.016 | Insertion of a 267bp fragment and deletion of a 469bp segment, amino aqcid substitutions at positions 14, 31, 36 and 56 |
| TC-18 | 12 | Amino acid substitutions at positions 6, 16, 59, 90, 97, 118, 172 |
| TC-165 | 16 | Amino acid substitutions at positions 55, 59, 83, 90, 103. |
| TC-232 | 64 | Amino acid substitutions at positions 24, 31 and a nonsense mutation at position 52 (Stop codon) |
| TC-243 | 16 | Amino acid substitutions at 59, 62, 75, 85 and 114 |
| TC-296 | 4 | Amino acid substitution at positions 59, 71 and 118 |
| TC-376 | >256 | Amino acid substitution at positions 31, 51, 59,118 and 172 |

# Chapter 8 Results: Cloning, Expression and Purification of *H. pylori* DNA Gyrase

## 8.1 Introduction

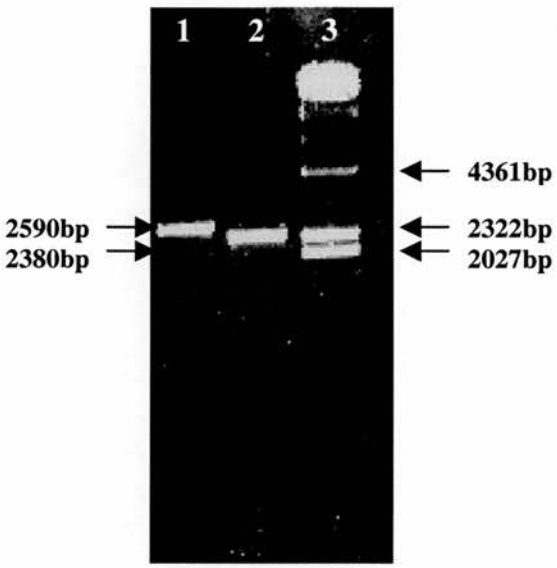
Bacterial DNA topoisomerases play an important role in cellular processes by controlling the topology states of DNA in DNA replication and transcription. DNA gyrase and topoisomerase IV are classified as type II topoisomerases. DNA gyrase, a heterotetramer protein, consist of two subunits, GyrA and GyrB, encoded by *gyrA* and *gyrB* (Swanberg and Wang, 1987). GyrA is responsible for the transient breakage and rejoining, whereas GyrB is responsible for ATPase activity, providing energy required for the supercoiling reaction. Topoisomerase IV, encoded by *parC* and *parE*, is a decatenating enzyme that acts during DNA replication (Kato *et al.*, 1990). DNA gyrase is a primary target of quinolones in gram-negative bacteria while topoisomerase IV is a primary target of quinolones in gram-positive bacteria (Chen *et al.*, 1996; Khodursky *et al.*, 1995; Ng *et al.*, 1996). In *H. pylori*, alterations in the QRDR of the *gyrA* gene have been reported to be associated with fluoroquinolone resistance by Moore *et al.* (1995). My study has also confirmed this finding (See Chapter 6). Since *parC* gene has not yet been identified in *H. pylori* genomic DNA (GenBank accession number NC_000915 and NC_000921), any contributions to the mechanism of quinolone resistance from topoisomerase IV cannot be quantified. This raised the question if DNA gyrase in *H. pylori* has topoisomerase IV activity to compensate for the absence of topoisomerase IV.

To characterise the DNA gyrase activity, cloning, expression and purification of DNA gyrase were determined. The *gyrA* and *gyrB* genes from a fluoroquinolone-sensitive *H. pylori* NCTC11637 were separately cloned and overexpressed in *E. coli* BL21 (DE3)pLysS. The individual subunits were purified and tested for DNA gyrase activity.

## 8.2 Cloning of PCR Fragments

The *gyrA* and *gyrB* genes of *H. pylori* NCTC11637 were amplified by PCR. A 2590-bp fragment was obtained from *gyrA* amplification and a 2380-bp fragment yielded from *gyrB* amplification (Figure 8.1). The PCR products of *gyrA* and *gyrB* were cloned separately into the expression vector pCR-T7/NT and then transformed to TOP 10F' competent cells. The positive clones were further identified by restriction analysis and DNA sequencing.

**Figure 8.1. PCR amplifications of *gyrA* and *gyrB* from *H. pylori* NCTC11637**




**Lane 1: *gyrA* PCR product**

**Lane 2: *gyrB* PCR product**

**Lane 3: Lambda/*Hin*dIII marker**

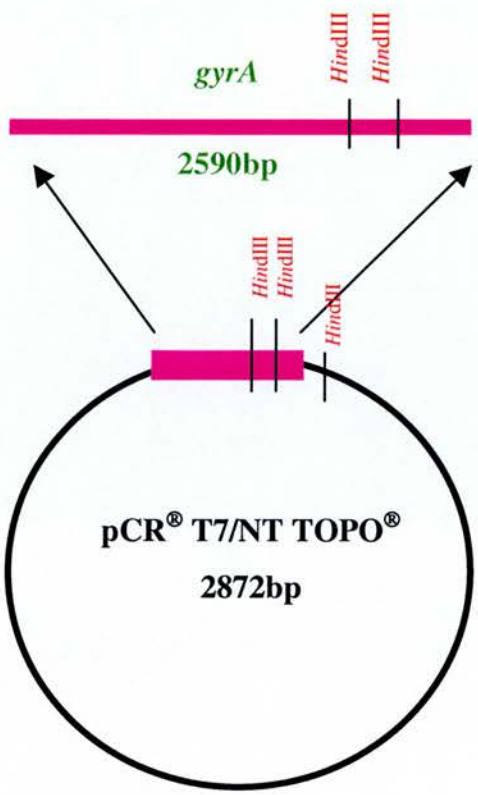
## 8.3 Analysis of Positive Clones

### 8.3.1 Restriction Analysis

Plasmids of the positive clones were analysed by restriction analysis and DNA sequencing to detect the orientation of the inserts. *Hin*dIII was used to cleave the plasmid pCR T7/NT harbouring a *gyrA* insert and a *gyrB* insert. The expected sizes of the restricted plasmid containing *gyrA* were 4462, 537, and 463 bp. The expected sizes of the restricted plasmid containing *gyrB* were 3660, 1023, 479 and 90bp. The restriction maps are shown in Figure 8.2. Restriction patterns of plasmids from clone 2A (*gyrA* clone) and clone 17B (*gyrB* clone) are shown in Figure 8.3 and 8.4. The results demonstrated expected *Hin*dIII fragments from both plasmids, suggesting that the plasmids pTC2A from clone 2A and pTC17B from clone 17B harboured *gyrA* and *gyrB* with the correct orientation, respectively.

**Figure 8.2. Restriction maps of plasmid pCR T7/NT TOPO containing *gyrA* and *gyrB*.** The *gyrA* and *gyrB* genes are indicated in pink and green boxes, respectively.

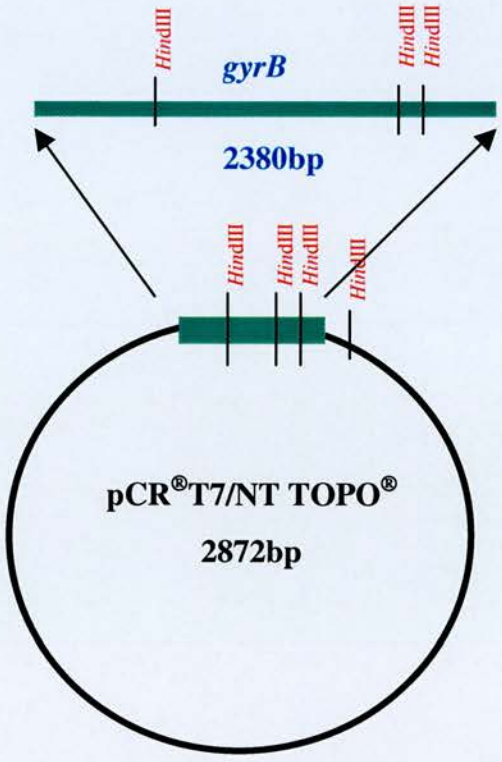
**(a) The map of pCR T7/NT TOPO containing a *gyrA* insert with *Hin*dIII restriction sites**




***Hin*dIII restriction site: Insert: position 1603 and 2140**

**: Vector: position 218**

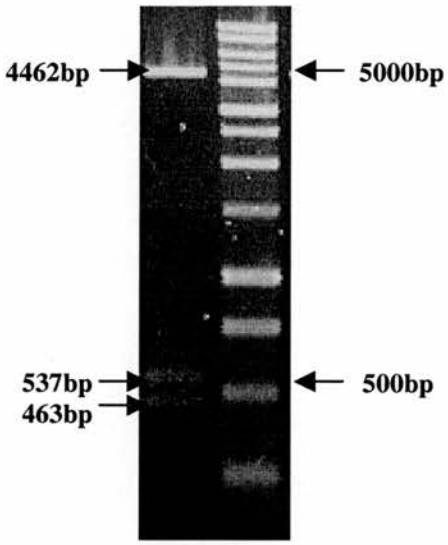
**(b) The map of pCR T7/NT TOPO containing a *gyrB* insert with *Hin*dIII restriction sites**




***Hin*dIII restriction site: Insert: position 801, 1824 and 1914**

**Vector: position 218**

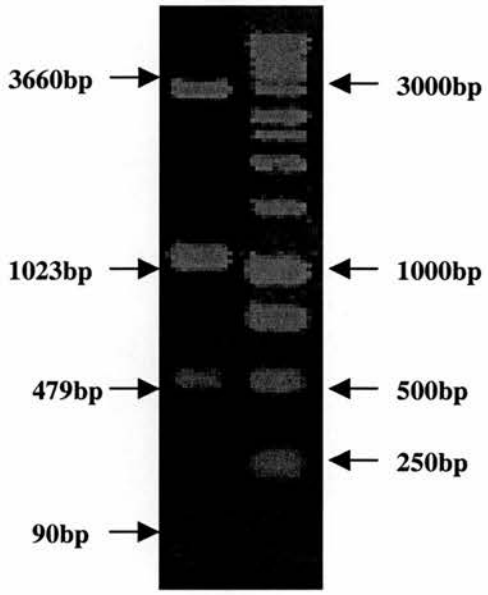
**Figure 8.3 Restriction patterns of plasmid pTC2A containing a *gyrA* insert.** Agarose gel shows restriction analysis of plasmid pTC2A from clone 2A. The *Hin*dIII fragments of 4459, 537 and 450bp indicated the correct orientation of the insert.




**Lane 1: *Hin*dIII fragments of plasmid pTC2A containing a *gyrA* insert**

**Lane 2: 1kb DNA ladder**

**Figure 8.4. Restriction pattern of plasmid pTC17B containing a *gyrB* insert.** Agarose gel shows restriction analysis of plasmid pTC17B from clone 17B. The *Hin*dIII fragments of 3660, 1023, 479 and 90bp indicate the correct orientation of the insert.




**Lane 1: *Hin*dIII fragments of plasmid pTC17B**

**Lane 2: 1kb DNA ladder**

### 8.3.2 Sequence Analysis

The plasmids pTC2A and pTC17B were sent to the Department of Haematology, Royal Infirmary, Edinburgh, Scotland for automated sequencing. The T7 sequencing primer (a vector-specific forward primer) was used to determine the orientation of the inserts and identify whether the inserts were in frame with the N-terminal tag. The sequence of pTC2A and pTC17B were compared the vector pCR T7/NT and shown in Figure 8.5 and 8.6. Sequence analysis demonstrated that both *gyrA* and *gyrB* inserts were in frame with the N-terminal tag and placed in the correct orientation.

The complete nucleotide sequences of *gyrA* and *gyrB* inserts were determined by automated DNA sequencing on the plasmid DNA templates, pTC2A and pTC17B. The sequence analysis showed that an open reading frame of 2322 and 2487 nucleotides coded for a polypeptide of 828 and 773 amino acids for *gyrA* and *gyrB*, respectively, with a calculated molecular mass of 92 kDa for GyrA and 86 kDa for GyrB. The complete sequences of *gyrA* and *gyrB* genes are show in Appendix II.

**Figure 8.5. Sequence analysis of pTC2A containing a *gyrA* insert.** The nucleotide sequence of pTC2A was compared with the vector pCR T7/NT. The partial nucleotide sequence of the *gyrA* insert in plasmid pTC2A is shown in bold and red. The yellow box indicates amino acid of the N-terminal tag. Polyhistidine region (6xHis) is indicated in bold. The grey box indicates the amino acid of the *gyrA* gene. Ribosome binding site is shown in green. Gaps in the sequence are indicated with dashes.

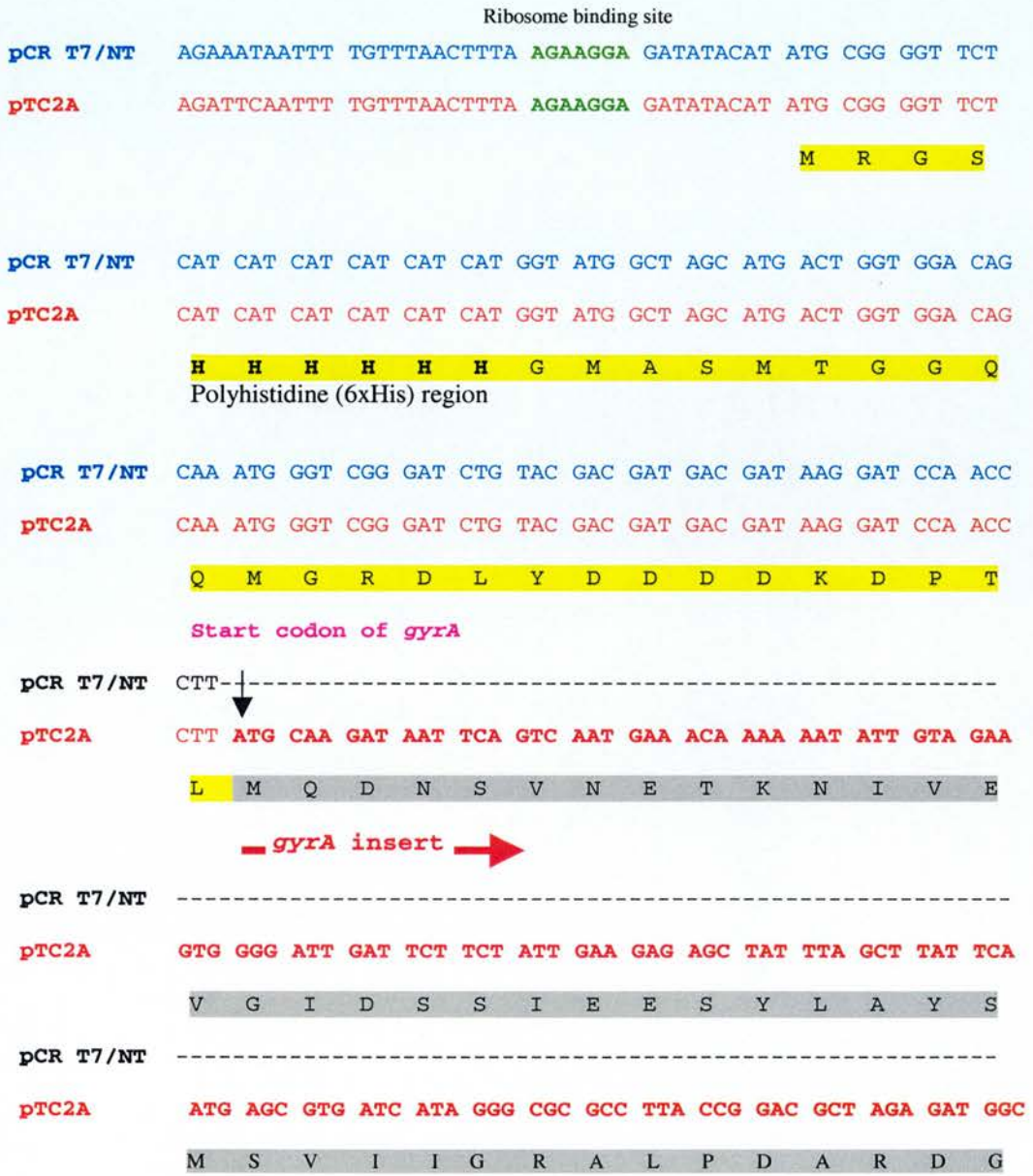
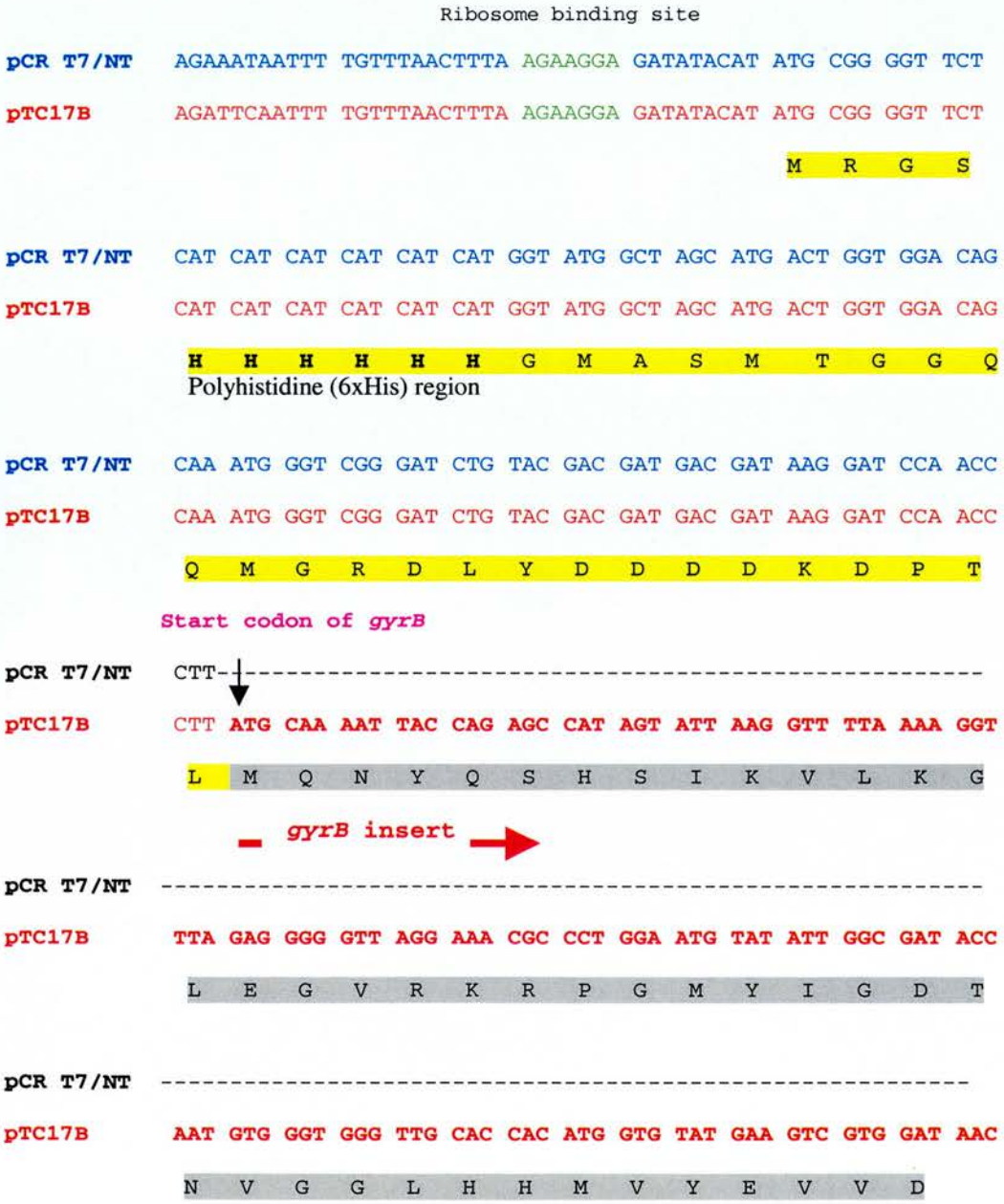


**Figure 8.6. Sequence analysis of pTC17B containing a *gyrB* insert.** The nucleotide sequence of pTC17B was compared with the vector pCR T7/NT. The partial nucleotide sequence of the *gyrB* insert in plasmid pTC17B is shown in bold and red. The yellow box indicates amino acid of the N-terminal tag. Polyhistidine region (6xHis) is indicated in bold. The grey box indicates the amino acid of the *gyrB* gene. Ribosome binding site is shown in green. Gaps in the sequence are indicated with dashes.



## 8.4 Expression of *gyrA* and *gyrB* of *H. pylori*

The plasmids pTCA2 and pTCB17 were overexpressed in *E. coli* BL21 (DE3)pLysS . After IPTG induction, the His-tagged proteins were detected by SDS-PAGE. The diagram of the His-tagged GyrA and GyrB are shown in Figure 8.7 and the SDS-PAGE analysis are shown in Figure 8.8. The expected sizes of the His-tagged GyrA and GyrB are 96kDa and 90kDa, respectively. SDS-PAGE analysis showed low-level expression of GyrA and GyrB. Although the expression conditions, including time, temperature and IPTG concentration, were optimised, the expression was still low. The proteins were expressed at 37°C with 1mM IPTG for 4 hours. As the bands of expected size of the His-tagged GyrA and GyrB were observed, the western blot analysis was further determined to confirm that these bands were the His-tagged proteins.

**Figure 8.7. Diagram of the His-tagged GyrA and GyrB.** GyrA and GyrB are indicated in pink and green boxes, respectively. Yellow box shows the N-terminal tag.

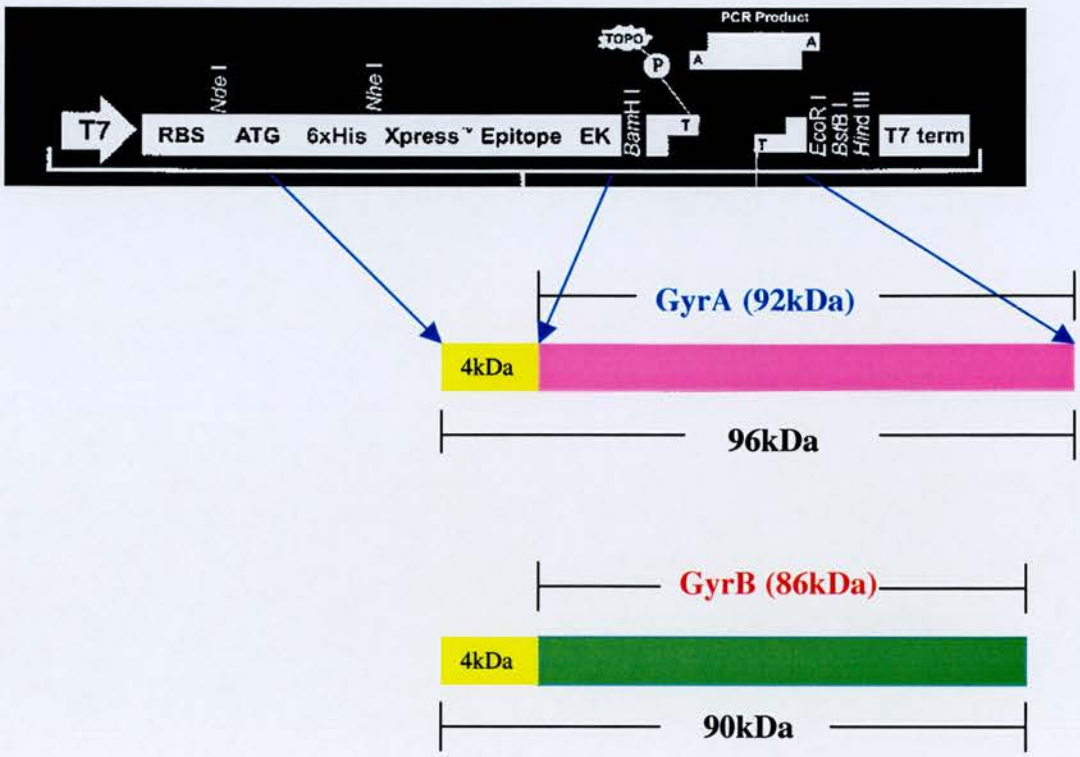
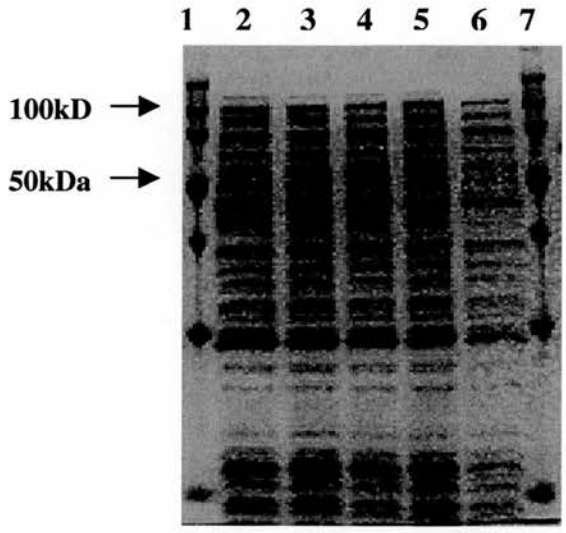


**Figure 8.8. SDS-PAGE analysis of GyrA (a) and GyrB (b) expressions.**

**(a) GyrA expression.**

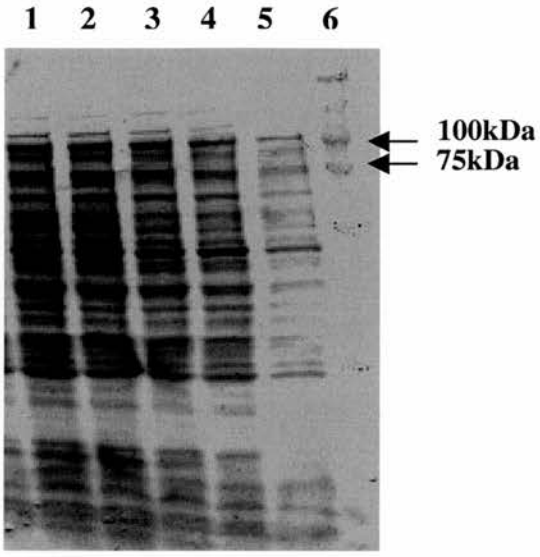



**Lane 1,7: MW marker**

**Lane 2-5: IPTG-induced cell lysate after 4, 3, 2 and 1 hours, respectively**

**Lane 6: Uninduced cell lysate**

**(b) GyrB expression.**




**Lane 1-4: IPTG-induced cell lysate after 4, 3, 2 and 1 hours, respectively.**

**Lane 5: Uninduced cell lysate**

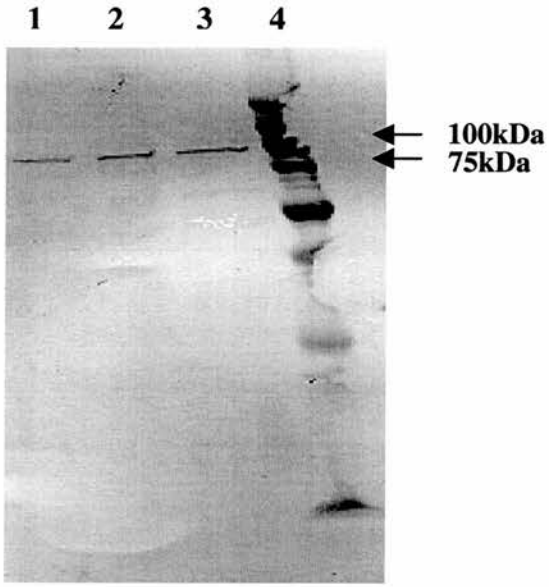
**Lane 6: MW marker**

## 8.5 Western Blot Analysis

To detect the expression of GyrA and GyrB fusion proteins, western blot analysis were determined. The antibody to 6xHis was used to detect the presence of the fusion proteins. The His tail is located at the N-terminal of the fusion protein. The results showed a single band for GyrA purification but two bands for GyrB purification (Figure 8.9). The molecular weights were estimated to be 96 kDa for GyrA and 90 kDa for GyrB, which correspond to the predicted amino acid plus 4kDa increased in the size of both proteins, derived from the N-terminal tag (See Figure 8.7). The unexpected band of GyrB was approximately 67kDa. This protein should be a truncated protein of GyrB as this protein was hybridised with the anti-6xHis-tagged antibody. Western blot analysis also showed that His-tagged GyrA and GyrB proteins were observed before the IPTG induction. This can explain why no distinct bands of the expected recombinant proteins were found on the SDS-PAGE gels after IPTG induction.

**Figure 8.9. Western blot analysis of GyrA and GyrB expressions.**

**(a) GyrA**



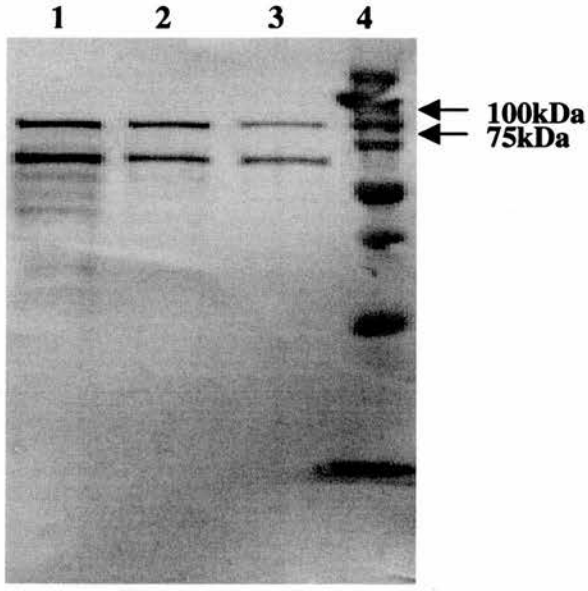

**Lane 1: Uninduced cell lysate**

**Lane 2: IPTG-induced cell lysate after 4 hours**

**Lane 3: IPTG-induced cell lysate after 2 hours**

**Lane 4: MW marker.**

**(b) GyrB**




**Lane 1: IPTG-induced cell lysate after 4 hours**

**Lane 2: IPTG-induced cell lysate after 2 hours**

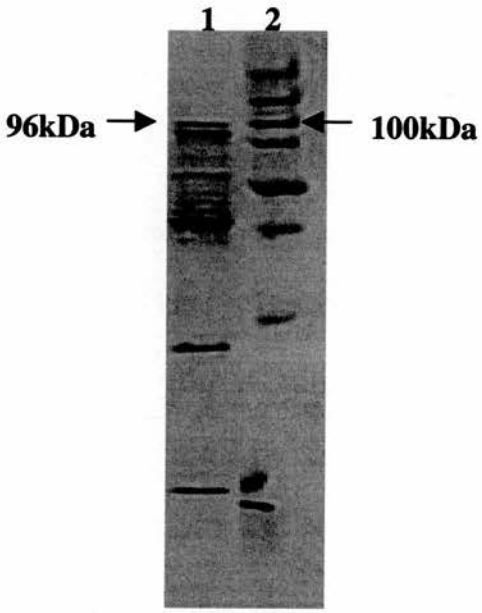
**Lane 3: Uninduced cell lysate**

**Lane 4: MW marker**

## 8.6 Protein Purification

GyrA and GyrB proteins were separately purified by affinity chromatography with ProBond resin (Nickel-charged Sepharose resin). The His-tagged proteins bound to the resin and were eluted with imidazole. SDS-PAGE analysis of the fractions eluted from the column was determined. The proteins with approximately expected size for GyrA and GyrB were found in the fractions. However, the results revealed that both recombinant GyrA and GyrB had low affinity for resin as they came off with contaminant proteins as shown in Figure 8.10 and 8.11. These partially purified GyrA and GyrB were used to further determine the activity of DNA gyrase as the time is limited for this study.

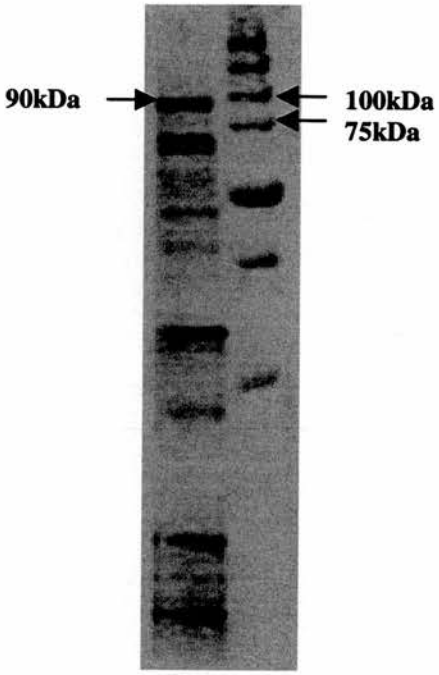
**Figure 8.10. GyrA purification**




**Lane 1: Purified GyrA**

**Lane 2: MW marker**

**Figure 8.11. GyrB purification**




**Lane 1: Purified GyrB**

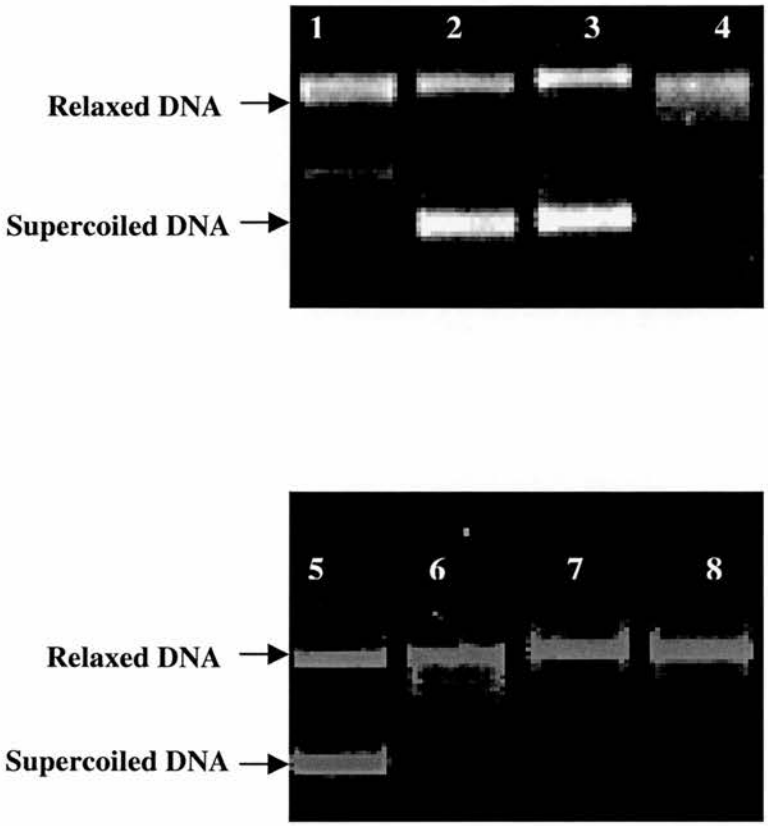
**Lane 2: MW marker**

## 8.7 DNA Supercoiling Assay

The partially purified GyrA and GyrB (Section 8.5) were assayed for the DNA supercoiling activity. The results are presented in Figure 8.12. No DNA supercoiling activity was detected from the protein preparations. The positive control derived from DNA gyrase of *E.coli* had the supercoiling activity. As the time is limited, the optimum condition for this assay could not be further determined.

These results show that the conditions were not optimal for supercoiling activity, although the positive control worked. Alteration of the relative protein concentrations did not improve the situation so it would be necessary to re-evaluate the individual polypeptides. This would have to be performed probably by N-terminal sequencing. Unfortunately, time and resources did not allow this to happen.

**Figure 8.12 Comparison of supercoiling activities of *H. pylori* DNA gyrase purified from NCTC11637 and *E.coli* DNA gyrase.** Relaxed pBR322 was incubated with either 1, 2 or 4 units of *E.coli* gyrase (Lane 1, 2 and 3) or no enzyme (Lane 4 and 6) or *H.pylori* gyrase partially purified from NCTC11637 (Lane 7 and 8). Lane 5: pBR322 (a mixture of supercoiled and relaxed DNA)



# Chapter 9 Discussion

Antimicrobial therapy has been widely used for *H. pylori* infection. As a single antibiotic fails to eradicate *H. pylori*, combinations of a proton pump inhibitor and antibiotics such as amoxycillin, clarithromycin, metronidazole and tetracycline are commonly used to eradicate *H. pylori.* Resistance to antibiotics is an important factor for treatment failure and primary resistance reduces the success of *H. pylori* eradication (Houben *et al.*, 1999). Susceptibility testing of *H. pylori* is, therefore, increasingly important. In this study, the prevalence and antibiotic susceptibility patterns of *H. pylori* against amoxycillin, ciprofloxacin, clarithromycin, erythromycin, tetracycline and metronidazole were investigated to identify the potential resistance problems in Scotland. The susceptibility testing of metronidazole was also evaluated examining variations in methodology on the outcome of susceptibility testing.

This study shows results on mutations in target proteins of fluoroquinolones, macrolides and metronidazole, which target DNA gyrase, 23S rRNA and oxygen-insensitive nitroreductase, respectively. The study of target protein alterations associated with antibiotic resistance provides a better understanding of resistance mechanisms. In addition, this thesis demonstrates cloning of DNA gyrase and attempted to express *H. pylori* DNA gyrase.

## 9.1 Antibiotic Susceptibility of *H. pylori*

This work is the first prospective study in Scotland, investigating the susceptibility patterns and prevalence of antibiotic resistance in *H. pylori*. As *H. pylori* is the major cause of peptic ulcers disease and a risk factor in gastric cancer, eradication with antimicrobial therapy has been recommended. Antibiotic resistance in *H. pylori* is of particular concern as it is the major factor in the failure of eradication regimens (Peitz *et al.*, 1999) and the prevalence of resistance varies between countries. Therefore, antibiotic susceptibility profiles in individual areas would allow the selection of adequate antibiotic treatment for successful *H. pylori* eradication.

Clarithromycin, metronidazole, amoxycillin and tetracycline are the antibiotics frequently used in the treatment regimens. Although *H. pylori* is susceptible to many antibiotics *in vitro*, resistance has been reported to most antimicrobials used. Tetracycline and clarithromycin resistance can be found in 5%-10%. Metronidazole resistance is more common and variable, ranging from 10% to >90% (Dunn *et al.*, 1997).

In this study $MIC_{50}$, $MIC_{90}$ of amoxycillin, ciprofloxacin, clarithromycin, erythromycin, tetracycline and metronidazole showed that amoxycillin was the most potent antibiotic against *H. pylori* as the $MIC_{90}$ was <0.016 mg/L and no resistance was found in all of the strains. For macrolides, clarithromycin showed high activity against *H. pylori* with the $MIC_{90}$ of 0.023mg/L, compared with the $MIC_{90}$ of 0.125mg/L for erythromycin.

Clarithromycin is the second potent antibiotic, followed by tetracycline and ciprofloxacin with the $MIC_{90}$ of 0.064mg/L, erythromycin ($MIC_{90}$ = 0.125mg/L) and metronidazole ($MIC_{90}$ = 0.75mg/L). The $MIC_{50}$ and $MIC_{90}$ values of all antibiotics are below the breakpoints, suggesting that these antibiotics have high activities against *H. pylori*. Metronidazole is the least active compared to other antibiotics tested. This result is similar to previous studies except that the $MIC_{90}$ of metronidazole ($MIC_{90}$ =0.75mg/L) obtained from this study is much lower than that of other reports ($MIC_{90}$ range of 16-128mg/L) (Lopez-Brea *et al.*, 1997; Megraud *et al.*, 1999; Piccolomini *et al.*, 1997; Wolle *et al.*, 2002). It is likely that the difference in the method determining metronidazole susceptibility is involved in the variations in the MICs. Pre-incubation under anaerobic conditions (See Chapter 4) was performed in this study.

In the present study, the prevalence of macrolide resistance is greater than other antibiotics, 9.1% (10/110) for erythromycin and 8.2% (9/110) for clarithromycin. This rate was higher than those in Sheffield, the UK (4.4%), the Netherlands (1.7%), Germany (2.2%), Ireland (4.5%) but lower than those in Spain (21%), Poland (23.5%) (Debets-Ossenkopp *et al.*, 1999; Dzierzanowska-Fangrat *et al.*, 2001; Lopez-Brea *et al.*, 2001; Parsons *et al.*, 2001; Wolle *et al.*, 2002; Xia *et al.*, 1996).

Cross-resistance among macrolides has been reported (Glupczynski and Burette, 1990; Xia *et al.*, 1996). In this study, all clarithromycin-resistant strains were resistant to erythromycin (MIC > 256mg/L). This suggests that the history of previous use of any macrolides should be taken into account when clarithromycin is prescribed for *H. pylori*

eradication. The increasing use of macrolide in clinical practice is also associated with the rise of clarthromycin resistance in *H. pylori*.

Clarithromycin, a newer macrolide with greater acid stability and higher blood and tissue level than erythromycin (Goldman *et al.*, 1994; Hardy *et al.*, 1988), is a potent component of most widely used regimens for *H. pylori* eradication. Resistance to clarithromycin will affect the cure rates of *H. pylori* and account for treatment failure. The increased consumption of macrolides is likely to influence the prevalence of clarithromycin resistance in *H. pylori*.

The prevalence of metronidazole resistance is highly variable. In developed countries, a European multicenter study reported that the overall metronidazole resistance rate was 27%, ranging from 16% in France to 42% in Norway (Megraud *et al.*, 1999). Parsons *et al.* al reported overall metronidazole resistance was 40% in Sheffield, UK (Parsons *et al.*, 2001). Resistance rates reported from the US was 37% (Osato *et al.*, 1999), the Netherlands was 21% (Debets-Ossenkopp *et al.*, 1999). Resistance rates are higher in developing countries; resistance rate in China is 78% (Wu *et al.*, 2000) and it can be as high as 90% in India (Mukhopadhyay *et al.*, 2000).

Resistance to metronidazole in this study is relative low (7.3%), comparing with other reports. The difference in methodology for the susceptibility testing of metronidazole (See Chapter 4) could explain the lower in the prevalence of metronidazole resistance in Scotland. Lack of standardisation of susceptibility tests makes it more difficult to

categorise a strain as susceptible or resistant. The consumption of metronidazole for the gynaecological or dental treatment may contribute to the increase in metronidazole resistance in developed countries. As metronidazole resistance is higher in developing countries, this may be caused by the common use of metronidazole in protozoa diseases and genital infection.

In this thesis, resistance to both metronidazole and clarithromycin was identified. Two of the 8 metronidazole-resistant isolates (25%) were resistant to clarithromycin. Double resistance to metronidazole and clarithromycin has been reported in other studies (Debets-Ossenkopp *et al.*, 1999; Osato *et al.*, 1999; Parsons *et al.*, 2001). In the UK, Parsons also found that 21 of the 36 macrolide-resistant isolates (58.3%) had metronidazole resistance (Parsons *et al.*, 2001). However, they may have shown a greater level of metronidazole because of the method that they used to test it. Dual resistance may be the consequence of the combination of therapy of clarithromycin and metronidazole.

Amoxycillin resistance is infrequent. No amoxycillin resistance was identified in this study. Similar results are reported by the studies in the Netherlands, Germany, Spain, the UK (Debets-Ossenkopp *et al.*, 1999; Parsons *et al.*, 2001; Wolle *et al.*, 2002) although 31% of amoxycillin-resistant strains isolated in Italy (Dore *et al.*, 1998) and 71.9% of isolates from China (Wu *et al.*, 2000) were reported. The reason for the high rate of amoxycillin resistance in Italy is unclear but in China, amoxycillin has been commonly used in the community in recent years.

Tetracycline resistance has been recently reported in one *H. pylori* strain in Australia (Midolo *et al.*, 1996). Piccolomini *et al.* showed that 6% of *H. pylori* strains isolated in Italy had tetracycline resistance (Piccolomini *et al.*, 1997). No resistance to tetracycline was observed in the studies in Germany, the Netherlands (Debets-Ossenkopp *et al.*, 1999; Wolle *et al.*, 2002). In the U.K, tetracycline resistance has been reported to be 0.5% (Parsons *et al.*, 2001). A similar result is obtained from this study, with the resistance rate of 0.9%. A report from China, where tetracycline resistance is used extensively as therapy for other infections, such as non-gonococcal urethritis, showed high rates of tetracycline resistance of 58.8% (Wu *et al.*, 2000).

Ciprofloxacin resistance is usually reported less than 1%. In this study, ciprofloxacin resistance is 1.8%. As ciprofloxacin is not currently used in the treatment regimens, the result suggests that ciproflaoxacin resistance in these strains may result from the use of fluoroquinolone for the treatment of other diseases. Unlike macrolides, the greatest concentration of fluoroquinolone use is to treat severe infections in the hospital, not in the community and so the exposure to fluoroquinolone has probably been limited (Megraud, 1997). Recently, fluoroquinolone resistance has been reported in the Netherlands with increasing resistance rate. Trovafloxacin resistance was 4.7%, suggesting that the resistance may be caused by the induction by other quinolones as trovafloxacin has not yet been introduced in the market in the country (Debets-Ossenkopp *et al.*, 1999).

The results from this study shows the importance of sensitivity testing before starting therapy as macrolide and metronidazole resistance is emerging in *H. pylori* isolated in Scotland. It would be beneficial for treatment outcome, especially when clarithromycin and metronidazole are prescribed. Inter-laboratory standardisation is also required for the accurate susceptibility profiles. This study does not demonstrate whether treatment has caused resistance, as there are no data on whether patients had previously been treated for *H. pylori*. However, they would provide a useful guide for appropriate eradication regimens for the patients.

## 9.2 Susceptibility Testing for Metronidazole

Treatment of *H. pylori* infection requires a combination of antimicrobial agents. Metronidazole is frequently used in the treatment regimens. The prevalence of metronidazole resistance varies between countries, ranging from 11-70% in developed countries and up to 90% in developing countries (Dunn *et al.*, 1997). Since *H. pylori* susceptibility testing has not yet been standardised, the variation may be the differences in the method determining susceptibility test.

Resistance to metronidazole has been reported to be associated with a significantly lower *H. pylori* eradication rate (Bell *et al.*, 1992; Buckley *et al.*, 1997; Burette *et al.*, 1992; Noach *et al.*, 1994; Rautelin *et al.*, 1992). It was demonstrated that triple therapy provided an eradication rate of 86-98% in patients with metronidazole-sensitive strains, however, eradication rate in the patients infected with metronidazole-resistant strains was still up to 68%, which is relatively high. These findings indicate that metronidazole still has the activity against some metronidazole-resistant strains. Cederbrant *et al.* (1992) demonstrated that significant decreases in metronidazole MIC were observed in metronidazole-resistant strains when there were pre-incubated under anaerobic conditions. Smith and Edwards showed that *H. pylori* NCTC11637 was resistant to metronidazole when tested under microaerophilic conditions, but became susceptible when the plates were pre-incubation under anaerobic conditions (Smith and Edwards, 1995).

The question is raised whether the reported resistant strains under microaerophilic conditions *in vitro* are true resistant strains *in vivo*. As metronidazole resistance affects the *H. pylori* eradication rate of metronidazole-including therapy, this study evaluated variations in methodology on the outcome of susceptibility testing, including anaerobic pre-incubation, media, size of inoculum and incubation time.

Pre-incubation under anaerobic conditions was shown to significantly decrease the MIC of metronidazole in most of the strains. The ratio between the MIC determined with and without pre-incubation ranged from 2.6 to 16,000 fold, except one strain (HP-91) had no change in the MIC (MIC >256mg/L). Only 6 strains showed less than 10-fold decreased in the MIC. The $MIC_{50}$ and $MIC_{90}$ decreased from 0.5 and >256mg/L to 0.016 and 0.19mg/L. This finding is in agreement with that reported by Cederbrant *et al* (1992). The result suggests that metronidazole is activated under anaerobic conditions by the reduction of a nitro group to a radical anion that damages the DNA (Edwards, 1993). The requirement of anaerobic pre-incubation may explain the high resistance rate of metronidazole reported by some centres. In this study, resistance to metronidazole was 28.6% (6/21) when tested under microaerophilic conditions, whereas the resistance rate was 4.7% (1/21) when tested under anaerobic conditions for 24 hours followed by microaerophilic conditions for 72 hours. This result showed a significant decrease in resistance levels when anaerobic pre-incubation was performed. It appears that anaerobic conditions are essential for resistant strains to restore resistant phenotype. This finding is in agreement with the findings by previous studies, which showed all metronidazole-resistant isolates became susceptible when pre-incubated for 24 hours

under anaerobic conditions (van Zwet *et al.*, 1995; Weel *et al.*, 1996). These findings also supports the hypothesis of Cederbrant *et al.* that resistant strains have lost the ability to reach a sufficient low redox potential to reduce a nitro group of metronidazole in microaerophilic environments, which is essential for reduction of metronidazole (Cederbrant *et al.*, 1992). Chatsuwan and Amyes (1999) suggested that anaerobic pre-incubation should be used for the determination of metronidazole susceptibility testing (Chatsuwan and Amyes, 1999).

In contrast, Abdi *et al.* showed that anaerobic pre-incubation had little effect on the estimation of prevalence of metronidazole resistance and appeared to have little advantage over microaerophilic incubation alone (Abdi *et al.*, 1999). The difference may be a large number of resistant strains were presented in the study. As the resistance rate when tested under microaerophilic conditions by E-test was 60%, comparing with 56% when tested with anaerobic pre-incubation.

A comparison was made contrasting the incubation of the MIC plates in an anaerobic jar and an anaerobic chamber, before transferring to microaerophilic conditions. Inconsistency in *H. pylori* growth was observed when the MIC plates were pre-incubated in an anaerobic chamber. *H. pylori* grew very well when an anaerobic jar was used to achieve anaerobic conditions, implicating that *H. pylori* requires high humidity for growth.

In this study, the effects of medium, inoculum size and incubation period were evaluated. It was demonstrated that *H. pylori* grew very well on Columbia agar with 7% horse blood, comparing with poor growth observed on Mueller-Hinton agar with 7% horse blood. The MICs were difficult to interpret when the inoculum size was 0.5 McFarland because of the poor growth but was clear and reproducible with higher inoculum (a No.3 McFarland standard). A similar observation has been reported by Hartzen *et al.* that higher inoculum size gave better results than the lower one (Hartzen *et al.*, 1997). After a 24-hour anaerobic pre-incubation, the MICs were easier to read when the plates were incubated under microaerophilic conditions for 72 hours.

In this study, the optimum conditions for metronidazole susceptibility testing of *H. pylori* were identified. In particular, it is imperative that pre-incubation of *H. pylori* under anaerobic conditions, in an anaerobic jar, is necessary to achieve accurate susceptibility results. Our interpretation is that these *H. pylori* strains are inherently sensitive to metronidazole. If the drug is pre-reduced, as it has to be for metronidazole sensitivity tests with other bacteria, then most *H. pylori* are extremely sensitive. It has been suggested that these bacteria are resistant under microaerophilic conditions. As there is no definite means of verifying whether the conditions used in the sensitivity test exactly match those at the site of infection, the conclusion is unwarranted. However, the clinical success of metronidazole suggests that most bacteria truly are sensitive to this drug as my sensitivity testing suggests.

## 9.3 Macrolide Resistance

Clarithromycin is active against *H. pylori*, even in the acidic environment of the stomach. This is the reason why clarithromycin is often used as an eradication regimen for *H. pylori* infection. However, development of resistance is predominantly a reason for therapy failure. In *E. coli* and some other bacteria, the most common macrolide resistance mechanism is methylation of the A2058 in 23S rRNA by methytransferases. The A2058 is the target of ribosomal methyltransferase, encoded by the *erm* gene and is also the macrolide-binding site (Vannuffel *et al.*, 1992; Weisblum, 1995). However, in *H. pylori*, point mutations in the 23S rRNA have been found to reduce drug binding to ribosome (Occhialini *et al.*, 1997) and consequently confers macrolide resistance (Hulten *et al.*, 1997; Occhialini *et al.*, 1997; Versalovic *et al.*, 1996). Similar findings were observed in *Mycobacterium* spp., *Mycoplasma pneumoniae* or *Propionibacterium* spp that mutations in the 23S rRNA conferred macrolide resistance (Ross *et al.*, 1997) (Lucier *et al.*, 1995; Meier *et al.*, 1994; Nash and Inderlied, 1995).

Initially, mutations conferring macrolide resistance in *H. pylori* were identified at positions 2058 and 2059 (Hartzen *et al.*, 1997) or positions 2143 and 2144 (Versalovic *et al.*, 1997) or positions 2514 and 2515 (Debets-Ossenkopp *et al.*, 1996) by different groups of investigators. After the study by Taylor *et al.*, positions correspond to *E. coli* bases 2058 and 2058 are considered as positions 2142 and 2143 in *H. pylori*, based on the structure of the gene in *H. pylori* (Taylor *et al.*, 1997). Five distinct point mutations have been reported to confer macrolide resistance in *H. pylori* isolates, i.e., G2115A,

G2141A, A2142G, A2142C and A2143G (Hulten *et al.*, 1997; Occhialini *et al.*, 1997; Stone *et al.*, 1997; Versalovic *et al.*, 1996). The A2142G and A2143G mutations are the most predominant among macrolide-resistant strains.

This study analysed the association between macrolide resistance and mutations in the peptidyl transferase region of the 23S rRNA gene in 10 macrolide-resistant isolates, nine of which were resistant to clarithromycin and cross-resistant to erythromycin (MIC of >256mg/L), suggesting the common target between macrolides. Mutations in the 23S rRNA were characterised by DNA sequencing. The MICs were correlated with the presence of alterations in the 23S rRNA. Point mutations were detected in 9 of the 10 macrolide-resistant isolates (90%). The majority of isolates (70%, 7/10) contained the A2143G mutation. The result is similar to the finding of Gracia-Arata *et al.*, Alarcon *et al.* and Domingo *et al.* (Alarcon *et al.*, 2000; Domingo *et al.*, 1998; Garcia-Arata *et al.*, 1999) but different from some other reports, which found mutation at A2142G in the majority of resistant isolates (Versalovic *et al.*, 1997; Wang and Taylor, 1998). Although mutation at A2142G and A2143G usually found in macrolide-resistant *H. pylori*, mutation at A2142G was not detected in this study. It has been demonstrated that high-level clarithromycin resistance is associated with the presence of the A2142G mutation in *H. pylori* (Alarcon *et al.*, 2000; Versalovic *et al.*, 1997). The absence of A2142G in this study may be because a small number of resistant isolates being studied and the MICs of most of the strains were not high. Only 3 clarithromycin-resistant isolates had the MIC of $\geq$ 32mg/L.

One isolate had mutation at A2143G and T2182C with the MIC of >256mg/L for both erythromycin and clarithromycin. These mutations were previously identified and reported that a C2182T mutation was not associated with clarithromycin resistance by site-directed mutagenesis (Wang *et al.*, 1998). In this study, a single mutation at T2182C was identified in one isolate with the clarithromycin MICs of 0.125mg/L and erythromycin MIC of >256mg/L. Therefore, this result supports the study by Wang *et al* and suggests that alternative resistance mechanisms play a role in this strain.

Mutations at C2195T and T1934C have not yet been reported. A C2195G was found in one isolate with the MIC of 8 and >256m/L for clarithromycin and erythromycin, respectively while a T1934C was presented with A2143G with the MIC of 32mg/L for clarithromycin and of >256mg/L for erythromycin. Further study is needed to be determined whether C2195T and T1934C mutations contribute to macrolide resistance. One isolate had very high macrolide MICs, erythromycin and clarithromycin MICs of >256mg/L but no mutation was found in this isolate. Other mechanisms must be involved in macrolide resistance in this isolate.

As a single point mutation is able to confer resistance to clarithromycin. This can explain why clarithromycin resistance develops at high frequency after eradication therapy. A widespread consumption of macrolides for respiratory or gastrointestinal tract infections may increase clarithromycin resistance in *H. pylori*.

## 9.4 Fluoroquinolone Resistance

Fluoroquinolones are potent antibiotics that have DNA gyrase and topoisomerase IV as their intracellular targets (Drlica, 1999). DNA gyrase introduces negative supercoiling of DNA and is required for DNA replication and RNA transcription (Maxwell, 1992). The primary target for Gram-negative bacteria is GyrA and for Gram positive is ParC (Gonzalez *et al.*, 1998; Kanematsu *et al.*, 1998; Mouneimne *et al.*, 1999; Nakano *et al.*, 1997; Yoshida *et al.*, 1990). Mutations conferring high-level quinolone resistance in many bacteria occur in the QRDR of the *gyrA* gene, which consist of amino acids 67-106 ((Maxwell, 1992; Yoshida *et al.*, 1990). Amino-acid substitutions in the QRDR in *E. coli* result in a reduction in quinolone binding to the gyrase-DNA complex (Maxwell, 1992). In *E. coli*, mutations at Serine-83 and Aspartic acid-87 give the greatest reduction in the susceptibility. Mutation of Serine-83 to a hydrophobic amino acid generally confers more resistance than mutation at Aspartic acid-87 (Khodursky *et al.*, 1995; Yoshida *et al.*, 1990). Moore *et al.* (1995) reported ciprofloxacin resistance in *H. pylori* isolated from the patients enrolled in a clinical trial evaluating the efficacy of ciprofloxacin for *H. pylori* eradication and determined the mechanism of resistance. It has been shown that resistance was associated with four classes of point mutations with amino-acid substitution at Asparagine-87 to Lysine, Alanine-88 to Valine, Aspartic acid-91 to Glycine or Asparagine, or Tyrosine and a double amino-acid substitutions at Aspartic acid-91 to Asparagine and Alanine-97 to Valine in the *gyrA* gene, encoding the A-subunit of DNA gyrase.

This study characterised *gyrA* gene from *H. pylori* to determine the mechanism of fluoroquinolone resistance in both *in vitro* and *in vivo*. The results demonstrate that ciprofloxacin resistance in *H. pylori* is due to mutations in the QRDR of *gyrA* gene with in agreement with the study by Moore *et al* (1995). This study also supports the existing data, which shows a common region in bacterial *gyrA* conferring fluoroquinolone resistance. Similar findings have also been found in other bacteria such as *E. coli* (Yoshida *et al.*, 1990) and *C. jejuni* (Wang *et al.*, 1993). Mutation at Serine-83 is the most common in *E. coli* and *C. jejuni*. In contrast to *H. pylori*, the most common amino-acid substitution in *H. pylori* reported is Aspartic acid-91 (Aspartic acid-87 in *E. coli*) (Moore *et al.*, 1995). This study also demonstrates that mutations at position 91 have been found in the majority of ciprofloxacin-resistant strains. All of the laboratory mutants had mutations either a single mutation at Aspartic acid-91 or a double amino-acid substitution at Aspartic acid-91 and either Glycine-85 or Asparagine-87. In addition, two of the three ciprofloxacin-resistant clinical isolates showed a single amino-acid substitution at Aspartic acid-91 and one had an amino-acid substitution at Asparagine-87 (amino acid 83 in *E. coli*). These results confirm the study by Moore *et al.* (1995).

As *parC* cannot be identified in the *H. pylori* genomic DNA (http;//www.ncbi.nlm.nih.gov/entrez/query.fcgi), the contribution of this gene toward fluoroquinolone resistance cannot be considered. This finding is similar to *C. jejuni*, which *parC* gene cannot be found in the genomic DNA, although Gibreel *et al.* reported mutation in *parC* at Arginine to Glutamine in *C. jejuni* (Gibreel *et al.*, 1998). It is likely that the *parC*

gene found in this study was not that of *C. jejuni*, as it was similar to *E. coli parC*, with 95% similarity.

The results obtained from this study reveal that *gyrA* mutation is primarily selected by ciprofloxacin. The second- and third-step mutants of HP-1, HP-4 and HP-7 did not show any additional mutations in their *gyrA* QRDR when the MICs were increased. Therefore, alternative mechanisms, such as active efflux, may play a role in ciprofloxacin resistance in *H. pylori*.

The ciprofloxacin-resistant mutants of HP-7 and NCTC 11916 had a double amino-acid substitution at amino acid 91 and 87 in their second-and third-step mutations and the mutant of HP-17 had a double amino-acid substitution at position 91 and 85 in its third-step mutation. These two types of double amino-acid substitutions found in this study have not been reported before in *H. pylori*. In the laboratory mutants, the MICs of ciprofloxacin for stains with two *gyrA* mutations (Mutants of HP–7, HP-17 and NCTC 11916) are not different from those for strains with only a single amino-acid substitution (Mutants of HP–1 and HP-4). Double amino-acid substitutions always found in the second- and third-step mutants with high-level resistance, but not in the first-step mutant, suggesting that two mutation sites in the *gyrA* gene confer high-level ciprofloxacin resistance in the laboratory mutants.

The results also demonstrated that a single amino acid change at Aspartic acid-91 to either Asparagine or Tyrosine (Mutants of HP-1 and HP-4, respectively) is responsible

for high-level ciprofloxacin resistance in *H. pylori*. These findings suggest that the loss of the acidic residual at position 91 is crucial for the emergence of fluoroquinolone resistance.

High-level resistance to ciprofloxacin was associated with either a single or double amino-acid mutations in the *gyrA* gene in laboratory mutants. A single mutation at either Aspartic acid-91 or Asparagine-87 was associated with ciprofloxacin-resistant in clinical isolates. Asparagine-87 in *H. pylori* is homologous to Serine-83 in *E. coli*. Mutations at Aspartic acid-91 may be comparable with the Aspartic acid-87 mutations in *E. coli gyrA*.

## 9.5 Metronidazole Resistance

Metronidazole resistance is one of the major causes of treatment failure as it is widely used in the eradication regimen. Mechanism of resistance is still unclear. Metronidazole has to enter bacterial cell in order that the nitro group of imidazole ring is reduced and form a hydroxylamine derivative, causing DNA damage and cell death (Edwards, 1993). Recently, Goodwin *et al.* (1998) identified the *rdxA* gene, encoding oxygen-insensitive NADPH nitroreductase, which can reduce metronidazole to its active form. It was demonstrated that the loss of this enzyme activity confers metronidazole resistance and showed that frameshift mutation, resulting in a truncated protein and missense mutations contributed to metronidazole resistance (Goodwin *et al.*, 1998). However, an intact *rdxA* gene product can be found in some metronidazole-resistant strains (Jenks *et al.*, 1999).

This study examined the role of *rdxA* in the development of metronidazole resistance in *H. pylori*. The results showed that 5 of the 6 metronidazole-resistant isolates had missense mutations and one isolate had a nonsense mutation, resulting in a stop codon and leading to a truncated protein. The results are similar to the study by Goodwin *et al.* that alterations in the *rdxA* gene were mostly due to missense mutations (Goodwin *et al.*, 1998). However, the mutations found in my study were not the same. In contrast to the study in the mouse model, a majority of resistant strains carried frameshift mutations, resulting in a stop codon (Jenks *et al.*, 1999). Tankovic *et al.* and Jenks *et al.* found that the majority of the frameshift mutations occurred within poly (A) tracts, suggesting that

slipped-strand mispairing may be an important mechanism in the regulation of gene expression (Jenks *et al.*, 1999; Tankovic *et al.*, 2000).

Solca *et al.* demonstrated amino acid substitutions were identified among both susceptible and resistant isolates. Various missense mutations were identified and 15 mutations were shared by both resistant and susceptible strains and no particular nucleotide mutation or amino acid substitution were associated to metronidazole resistance and there was no specific cluster associated with the resistance phenotypes in the phylogenic analysis (Solca *et al.*, 2000). Similar to the work by Solca *et al.*, the present study showed 20 different amino-acid changes (positions 6, 16, 24, 31, 51, 52, 55, 59, 62, 71, 75, 83, 85, 90, 97, 103, 114, 118 and 172) in *rdxA* of metronidazole-resistant strains, comparing with *rdxA* of *H. pylori* 26695. This study also demonstrated that the majority of missense mutations were found in susceptible strains when compared the result with other reports and there is no correlation between metronidazole MIC and either type or position of mutation in the *rdxA* gene.

Both *H. pylori* NCTC11637 and NCTC11916, which are metronidazole-sensitive strains, identified by our metronidazole-susceptibility testing, had three amino-acid substitutions and nonsense mutations at position 73, leading to a stop codon which was the result of deletions of a 469-bp segment and insertions of a 267-bp fragment. The insert is closely matched to the vacuolating cytotoxic gene and cag I pathogenicity island of *H. pylori* by BLAST search. The insert carried 41bp and 33bp at the left and right ends, closely matched to the left and right ends of IS*605*. This variant of IS*605* was

found at the right end of *cag* pathogenicity island in *H. pylori* strain CCUG 17874 (Censini *et al.*, 1996). My finding is different from the work by Debets-Ossenkopp *et al* in that *rdxA* of the *H. pylori* NCTC11637 had a nonsense mutation at position 83 instead of at position 73, identified by this study (Debets-Ossenkopp *et al.*, 1999).

The present study demonstrated that inactivation of the *rdxA* gene is not the sole contributor to the mechanism of metronidazole resistance. Only one of the six metronidazole-resistant isolates had amino acid substitution, causing a premature stop codon leading to a truncated RdxA and the finding that two metronidazole-sensitive strains had truncated RdxA, due to deletion and insertion in the *rdxA* gene. In addition, amino acid substitutions found in the resistant strains are also present in the sensitive strains, identified by other studies, and the *rdxA* genes in metronidazole-sensitive *H. pylori* strains differ by approximately 5% at the nucleotide level (Goodwin *et al.*, 1998). These findings suggest that mutations in the *rdxA* gene do not always confer metronidazole resistance and alternative mechanisms of resistance exist in *H. pylori*. Other mechanisms of resistance may be related to diminished uptake or active efflux; more efficient DNA repair; enhanced scavenging of toxic oxygen radicals and the loss of other enzyme involved in reduction of metronidazole to its active form.

## 9.6 Cloning, Expression and Purification of *H. pylori* DNA Gyrase

DNA gyrase introduces negative supercoils into covalent closed DNA by using the free energy derived from ATP hydrolysis. It is a heterotetramer, composed of GyrA and GyrB proteins, which are encoded by *gyrA* and *gyrB*, respectively. DNA gyrase activity of *H. pylori* has not yet been reported (Reece and Maxwell, 1991). This study cloned, overexpressed and purified GyrA and GyrB proteins to characterise the DNA gyrase activity in *H. pylori*. As *parC* has not yet been identified, characterisation of the DNA gyrase may explain the absence of topoisomerase IV in *H. pylori*.

In the present study, the complete nucleotide sequence of DNA gyrase of *H. pylori* NCTC 11637 was determined. The *gyrA* and *gyrB* genes were compared to *H. pylori* and other bacteria, confirming that the sequences are definitely *gyrA* and *gyrB* by BLAST search. The sequencing analysis revealed that *gyrA* had an open reading frame of 2487bp, coding for 828 amino acids. The open reading frame of *gyrB* of 2322bp coded for 773 amino acids. It was shown that GyrA and GyrB had a calculated molecular mass of 92kDa and 86kDa, respectively. In *E. coli*, GyrA and GyrB protein consist of 875 and 804 amino acids, with the molecular mass of 97 and 90kDa, respectively (Reece and Maxwell, 1991)

The *gyrA* and *gyrB* genes were compared to *H. pylori* and other bacteria, confirming that the sequences are definitely *gyrA* and *gyrB* by BLAST search. There is approximately

95-96% similarity in both *gyrA* and *gyrB* at the nucleotide levels. The DNA sequence of *gyrA* is similar to that of *H. pylori* UC 763, accession No. L29481 (Moore *et al.*, 1995) and the sequences from complete genome of *H. pylori* 26695 and J99 from GenBank (Accession No. AE000583 and AE001496, respectively). The results demonstrated that the *gyrA* and *gyrB* genes are diverse among *H. pylori* strains, as there was 5-6% difference between *H. pylori* strains.

In this study, the predicted GyrA for *H. pylori* NCTC11637 has one more amino-acid residue than the sequence of *H. pylori* 26695 and two more residues for *H. pylori* UC 763. *H. pylori* NCTC11637 has the same number of amino acids (828 amino acids) as that of *H. pylori* J99. Similarly, there is 5-6% difference in the GyrB, when compared with the *H. pylori* sequences in the GenBank. The predicted GyrB for *H. pylori* for *H. pylori* NCTC11637 has 773 amino acid residues, similar to both *H. pylori* 26695 and J99.

Compared to other bacteria, amino-acid sequence of *H. pylori* GyrA showed 59% identity with *C. fetus* and *C. jejuni*, 52% with *B. subtilis*, 45% with *E. coli* and *V. cholerae*. The GyrB protein of *H. pylori* had 92-93% identity with *H. pylori*, 55-56% with *C. jejuni* and 40-41% with *E. coli*, *V. cholerae*, *H. influenzae* and *Salmonella* spp. Similar to other bacteria, the active site of GyrA, Tyrosine-122, which involves in the DNA breakage and rejoining activities in *E. coli* (Horowitz and Wang, 1987), is found to be conserved in *H. pylori*. In *E. coli*, it has been reported that amino acid residues such as Tyrosine-5, Asparagine-46, Aspartic acid-73, Lysine-103, Tyrosine-109,

Glutamine-335, and Lysine-337 involve in ATP binding (Wigley *et al.*, 1991). All these residues are also conserved in *H. pylori* and other bacteria.

The GyrA and GyrB proteins were overexpressed after confirmation that the *gyrA* and *gyrB* inserts were in frame with the N-terminal tag and placed in the correct orientation in the expression vectors. The proteins were separately purified with affinity chromatography. The results of this study showed that no supercoiling activity could be detected on relaxed DNA exposed to a combination of GyrA and GyrB proteins. The degree of purity of the proteins could affect the supercoiling activity, as these proteins were partially purified. In addition, GyrA and GyrB (His-Tagged proteins) contained the N-terminal tag, which could certainly interfere with protein folding. Therefore, the removal of the N-terminal tag may improve the enzyme activity and the re-evaluation of A and B proteins need to be determined by N-terminal sequencing. As the time and resources are limited, this investigation needs much further study. However, it is very likely that the DNA gyrase of *H. pylori* must undertake the decatenation role normally associated with DNA topoisomerase IV.

# Chapter 10 Conclusions

Antibiotic susceptibility patterns of *H. pylori* strains isolated in Scotland against amoxycillin, ciprofloxacin, clarithromycin, erythromycin, tetracycline and metronidazole revealed that the $MIC_{50}$ and $MIC_{90}$ values of all antibiotics are below the breakpoints, indicating that these antibiotics have high activities against *H. pylori.* Amoxycillin is the most active compound and metronidazole is the least active compared to other antibiotics tested. Although, many antibiotics found to be active *in vitro*, antibiotic resistance to *H. pylori* is emerging. In this study, the prevalence of macrolide resistance is greater than other antibiotics, 9.1% (10/110) for erythromycin and 8.2% (9/110) for clarithromycin. Resistance to metronidazole is relative low (7.1%, 8/110), compared with other reports but this largely reflects differences in methodology for the susceptibility testing of metronidazole. Resistance to tetracycline with intermediate resistance was found in one isolate (0.9%). Ciproflaxacin resistance was found in 1.8% (2/110). No amoxycillin resistance was detected. The results from this study suggest the importance of sensitivity testing before eradication therapy as resistance to macrolide and metronidazole is emerging in *H. pylori* isolated in Scotland.

The method determining metronidazole susceptibility is involved in the variations in the MICs. This present study showed that a 24-hour pre-incubation under anaerobic conditions is necessary to achieve accurate susceptibility results. Activation of

metronidazole requires anaerobic environment. Susceptibility testing of metronidazole under microaerophilic conditions elevates the MICs.

This thesis demonstrated that mechanism of macrolide resistance was associated with alterations in the peptidyl transferase region of the 23S rRNA. Point mutations were detected in 9 of the 10 macrolide-resistant isolates (90%). The A2143G mutation was found in the majority of isolates (70%, 7/10). Cross-resistance between erythromycin and clarithromycin was demonstrated, suggesting the common target of macrolides.

High-level resistance to ciprofloxacin was found to be associated with two mutation sites in the *gyrA* gene at Aspartic acid-91 and either Glycine-85 or Asparagine-87 in laboratory mutants. The results also demonstrated that a single amino acid change at Aspartic acid-91 to either Asparagine or Tyrosine is responsible for high-level ciprofloxacin resistance in *H. pylori*, suggesting that the loss of the acidic residual at position 91 is crucial for the emergence of fluoroquinolone resistance. Mutation at Aspartic acid-91 to Asparagine was also found in ciprofloxacin resistant clinical isolates.

This study analysed the role of the *rdxA* gene conferring metronidazole resistance. The results suggest that mutations in the *rdxA* gene do not always confer metronidazole resistance and alternative mechanisms, such as reduced drug uptake, active efflux, more efficient DNA repair; enhanced scavenging of toxic oxygen radicals and the loss of other enzymes involved in reduction of metronidazole to its active form.

The complete *gyrA* and *gyrB* genes were separately cloned into pCR T7/NT vector. The sequencing analysis revealed that *gyrA* had an open reading frame of 2484bp, coding for 828 amino acids and the open reading frame of *gyrB* of 2322bp coded for 773 amino acids. GyrA and GyrB had a calculated molecular mass of 92kDa and 86kDa, respectively. It was found that the *gyrA* and *gyrB* genes are diverse among *H. pylori* strains as there was 5-6% difference between *H. pylori* strains. Unfortunately, the attempt to demonstrate DNA gyrase activity from the DNA gyrase subunit preparations was not successful after overexpression and purification of GyrA and GyrB. The results showed that supercoiling activity could not be detected on relaxed DNA exposed to a combination of GyrA and GyrB proteins. As they are partially purified, the effect of the impurity of the proteins could hinder the supercoiling activity. In addition, the N-terminal tag could interfere protein folding. As the time and resource are limited, this investigation needs much further study.

# Appendix I

| | | MIC (mg/L) | | | | | |
|---|---|---|---|---|---|---|---|
| NO | Strain | Amoxycillin | Ciprofloxacin | Clarithromycin | Erythromycin | Tetracycline | Metronidazole |
| 1 | TC-2 | <0.016 | 0.008 | <0.016 | 0.023 | 0.016 | <0.016 |
| 2 | TC-4 | <0.016 | 0.016 | 0.047 | <0.016 | <0.016 | <0.016 |
| 3 | TC-7 | <0.016 | 0.012 | <0.016 | <0.016 | <0.016 | <0.016 |
| 4 | TC-8 | <0.016 | 0.004 | <0.016 | <0.016 | <0.016 | <0.016 |
| 5 | TC-12 | <0.016 | 0.023 | <0.016 | <0.016 | 1 | <0.016 |
| 6 | TC-13 | <0.016 | 0.012 | <0.016 | <0.016 | <0.016 | <0.016 |
| 7 | TC-14 | <0.016 | 0.012 | <0.016 | 0.023 | <0.016 | <0.016 |
| 8 | TC-18 | 0.047 | 0.023 | >256 | >256 | 0.016 | 12 |
| 9 | TC-19 | <0.016 | 0.012 | 1.5 | >256 | 0.016 | <0.016 |
| 10 | TC-24 | 0.023 | 0.012 | >256 | >256 | 0.023 | 24 |
| 11 | TC-33 | <0.016 | 0.023 | <0.016 | 0.016 | 0.032 | 0.016 |
| 12 | TC-34 | <0.016 | 0.003 | <0.016 | <0.016 | 0.016 | <0.016 |
| 13 | TC-35 | <0.016 | 0.023 | <0.016 | 0.032 | 0.032 | <0.016 |
| 14 | TC-39 | <0.016 | 0.023 | 0.016 | 0.023 | 0.023 | <0.016 |
| 15 | TC-40 | <0.016 | 0.032 | 32 | >256 | 0.016 | <0.016 |
| 16 | TC-44 | <0.016 | 0.008 | <0.016 | 0.016 | <0.016 | <0.016 |
| 17 | TC-45 | <0.016 | 0.008 | <0.016 | 0.023 | 0.016 | <0.016 |
| 18 | TC-51 | <0.016 | 0.012 | <0.016 | <0.016 | 0.016 | <0.016 |
| 19 | TC-53 | <0.016 | 0.003 | <0.016 | <0.016 | <0.016 | <0.016 |

| | | MIC (mg/L) | | | | | |
|---|---|---|---|---|---|---|---|
| NO | Strain | Amoxycillin | Ciprofloxacin | Clarithromycin | Erythromycin | Tetracycline | Metronidazole |
| 20 | TC-56 | <0.016 | 0.012 | <0.016 | 0.023 | <0.016 | 0.016 |
| 21 | TC-57 | <0.016 | 0.032 | <0.016 | 0.032 | 0.023 | <0.016 |
| 22 | TC-75 | <0.016 | 0.032 | <0.016 | 0.016 | 0.016 | <0.016 |
| 23 | TC-78 | 0.016 | 0.023 | 6 | >256 | <0.016 | 0.016 |
| 24 | TC-83 | <0.016 | 0.047 | <0.016 | 0.023 | 0.032 | 0.023 |
| 25 | TC-97 | <0.016 | 0.032 | <0.016 | 0.016 | 0.023 | 0.016 |
| 26 | TC-107 | <0.016 | 0.064 | 0.016 | 0.125 | 0.094 | <0.016 |
| 27 | TC-110 | <0.016 | 0.047 | <0.016 | 0.032 | 0.032 | 0.064 |
| 28 | TC-111 | <0.016 | 0.032 | 0.016 | 0.064 | 0.016 | <0.016 |
| 29 | TC-115 | <0.016 | 0.047 | 0.016 | 0.023 | 0.094 | 0.047 |
| 30 | TC-116 | <0.016 | 0.032 | <0.016 | 0.032 | 0.047 | <0.016 |
| 31 | TC-120 | <0.016 | 0.064 | 0.016 | 0.064 | 0.064 | <0.016 |
| 32 | TC-127 | <0.016 | 0.016 | <0.016 | 0.023 | <0.016 | <0.016 |
| 33 | TC-130 | <0.016 | 0.19 | <0.016 | 0.047 | 0.047 | 0.016 |
| 34 | TC-131 | <0.016 | 0.064 | <0.016 | 0.094 | 0.064 | <0.016 |
| 35 | TC-140 | <0.016 | 0.047 | 3 | >256 | 0.023 | <0.016 |
| 36 | TC-142 | <0.016 | 0.047 | <0.016 | <0.016 | <0.016 | 0.125 |
| 37 | TC-144 | 0.016 | 0.016 | 0.016 | 0.047 | 0.016 | <0.016 |
| 38 | TC-145 | <0.016 | 0.032 | <0.016 | 0.023 | 0.023 | <0.016 |

| | | MIC (mg/L) | | | | | |
|---|---|---|---|---|---|---|---|
| NO | Strain | Amoxycillin | Ciprofloxacin | Clarithromycin | Erythromycin | Tetracycline | Metronidazole |
| 39 | TC-147 | <0.016 | 0.023 | <0.016 | 0.023 | 0.016 | <0.016 |
| 40 | TC-148 | 0.016 | 0.032 | <0.016 | 0.064 | 0.032 | 0.25 |
| 41 | TC-154 | <0.016 | 0.023 | <0.016 | 0.047 | 0.032 | 0.016 |
| 42 | TC-157 | <0.016 | 0.032 | <0.016 | 0.125 | 0.016 | 0.064 |
| 43 | TC-161 | 0.016 | 0.032 | <0.016 | 0.016 | 0.016 | <0.016 |
| 44 | TC-162 | <0.016 | 0.047 | <0.016 | 0.032 | 0.032 | <0.016 |
| 45 | TC-164 | <0.016 | 0.008 | <0.016 | <0.016 | 0.023 | >256 |
| 46 | TC-165 | 0.016 | 0.004 | 0.016 | 0.023 | 0.023 | 12 |
| 47 | TC-169 | <0.016 | 0.023 | <0.016 | 0.016 | 0.016 | 0.016 |
| 48 | TC-173 | <0.016 | 0.023 | <0.016 | 0.016 | <0.016 | 0.023 |
| 49 | TC-175 | <0.016 | 0.032 | 0.125 | >256 | 0.064 | 0.023 |
| 50 | TC-177 | <0.016 | 0.032 | <0.016 | 0.023 | <0.016 | <0.016 |
| 51 | TC-180 | 0.023 | 0.047 | <0.016 | 0.094 | 0.064 | 0.016 |
| 52 | TC-185 | <0.016 | 0.032 | 24 | >256 | 0.032 | 0.19 |
| 53 | TC-196 | 0.016 | 0.125 | 0.016 | 0.094 | 0.064 | 0.75 |
| 54 | TC-202 | <0.016 | 0.032 | <0.016 | 0.047 | 0.032 | <0.016 |
| 55 | TC-204 | <0.016 | 0.016 | <0.016 | 0.047 | <0.016 | 0.094 |
| 56 | TC-208 | <0.016 | 0.032 | 0.016 | 0.094 | 0.032 | 0.25 |
| 57 | TC-212 | 0.016 | 0.064 | 0.016 | 0.032 | 0.032 | 0.5 |

| | | MIC (mg/L) | | | | | |
|---|---|---|---|---|---|---|---|
| NO | Strain | Amoxycillin | Ciprofloxacin | Clarithromycin | Erythromycin | Tetracycline | Metronidazole |
| 58 | TC-214 | <0.016 | 0.032 | <0.016 | 0.016 | 0.016 | 0.25 |
| 59 | TC-215 | <0.016 | 0.016 | 3 | >256 | 0.032 | <0.016 |
| 60 | TC-220 | 0.016 | 0.064 | <0.016 | <0.016 | 0.023 | 0.75 |
| 61 | TC-223 | <0.016 | 0.032 | <0.016 | 0.032 | 0.094 | 0.064 |
| 62 | TC-224 | <0.016 | 0.012 | <0.016 | <0.016 | 0.016 | 0.25 |
| 63 | TC-225 | <0.016 | 0.032 | 0.016 | 0.032 | 0.032 | <0.016 |
| 64 | TC-232 | <0.016 | 0.094 | <0.016 | <0.016 | 0.032 | 64 |
| 65 | TC-234 | 0.032 | 0.064 | <0.016 | <0.016 | 0.125 | 0.023 |
| 66 | TC-242 | <0.016 | 0.032 | 0.023 | 0.064 | 0.032 | 0.047 |
| 67 | TC-243 | <0.016 | 0.008 | <0.016 | <0.016 | <0.016 | 16 |
| 68 | TC-246 | <0.016 | 16 | <0.016 | 0.032 | 0.032 | <0.016 |
| 69 | TC-273 | <0.016 | 0.047 | <0.016 | 0.094 | <0.016 | 1 |
| 70 | TC-283 | <0.016 | 0.023 | 0.016 | 0.094 | 0.032 | 1 |
| 71 | TC-284 | <0.016 | 0.032 | 8 | >256 | <0.016 | 0.094 |
| 72 | TC-292 | <0.016 | 0.023 | <0.016 | 0.094 | 0.016 | 1.5 |
| 73 | TC-294 | 0.016 | 0.023 | <0.016 | 0.094 | 0.032 | 0.094 |
| 74 | TC-296 | 0.023 | 0.032 | 0.016 | 0.032 | 0.094 | 4 |
| 75 | TC-300 | <0.016 | 0.016 | <0.016 | 0.023 | 0.016 | 0.047 |
| 76 | TC-304 | <0.016 | 0.032 | <0.016 | 0.023 | 0.19 | 0.016 |

| | | MIC (mg/L) | | | | | |
|---|---|---|---|---|---|---|---|
| NO | Strain | Amoxycillin | Ciprofloxacin | Clarithromycin | Erythromycin | Tetracycline | Metronidazole |
| 77 | TC-307 | <0.016 | 0.032 | <0.016 | 0.023 | 0.016 | 0.016 |
| 78 | TC-318 | <0.016 | 0.064 | 0.016 | 0.032 | 0.016 | 0.016 |
| 79 | TC-320 | <0.016 | 0.047 | <0.016 | 0.016 | 0.023 | 0.25 |
| 80 | TC-321 | <0.016 | 0.064 | <0.016 | 0.023 | 0.023 | 0.032 |
| 81 | TC-324 | <0.016 | 0.012 | <0.016 | 0.016 | 0.016 | <0.016 |
| 82 | TC-327 | <0.016 | 0.047 | <0.016 | 0.032 | 0.047 | 0.032 |
| 83 | TC-332 | <0.016 | 0.047 | <0.016 | 0.047 | 0.032 | 0.047 |
| 84 | TC-335 | <0.016 | 0.016 | <0.016 | 0.023 | 0.016 | 0.032 |
| 85 | TC-338 | <0.016 | 0.032 | <0.016 | 0.032 | 0.023 | 0.023 |
| 86 | TC-339 | <0.016 | 0.023 | <0.016 | 0.032 | <0.016 | 0.023 |
| 87 | TC-340 | <0.016 | 0.047 | <0.016 | <0.016 | <0.016 | 0.19 |
| 88 | TC-342 | <0.016 | 0.032 | 0.016 | 0.016 | <0.016 | 0.064 |
| 89 | TC-344 | <0.016 | 0.047 | <0.016 | <0.016 | 0.064 | 0.016 |
| 90 | TC-361 | <0.016 | 0.047 | <0.016 | 0.032 | 0.032 | 0.064 |
| 91 | TC-365 | <0.016 | 0.094 | <0.016 | 0.023 | 0.047 | 0.023 |
| 92 | TC-370 | <0.016 | 0.125 | <0.016 | 0.023 | 0.023 | 0.023 |
| 93 | TC-376 | <0.016 | 0.008 | <0.016 | <0.016 | <0.016 | >256 |
| 94 | TC-392 | <0.016 | 0.032 | <0.016 | 0.023 | 0.023 | <0.016 |
| 95 | TC-395 | 0.016 | 0.094 | <0.016 | 0.032 | 0.023 | 0.047 |

| | | MIC (mg/L) | | | | | |
|---|---|---|---|---|---|---|---|
| NO | Strain | Amoxycillin | Ciprofloxacin | Clarithromycin | Erythromycin | Tetracycline | Metronidazole |
| 96 | TC-402 | <0.016 | 0.023 | <0.016 | <0.016 | <0.016 | 0.023 |
| 97 | TC-404 | <0.016 | 0.032 | <0.016 | 0.023 | 0.016 | <0.016 |
| 98 | TC-405 | <0.016 | 0.064 | <0.016 | <0.016 | 0.016 | 0.064 |
| 99 | TC-409 | <0.016 | 0.004 | <0.016 | 0.032 | <0.016 | <0.016 |
| 100 | TC-413 | <0.016 | >32 | <0.016 | 0.023 | 0.016 | <0.016 |
| 101 | TC-415 | 0.016 | 0.023 | <0.016 | 0.032 | <0.016 | 0.016 |
| 102 | TC-419 | 0.016 | 0.064 | 0.023 | 0.064 | 0.023 | 0.5 |
| 103 | TC-421 | <0.016 | 0.023 | <0.016 | 0.023 | <0.016 | 0.016 |
| 104 | TC-429 | <0.016 | 0.032 | <0.016 | 0.023 | 0.016 | 0.064 |
| 105 | TC-430 | <0.016 | 0.064 | 0.023 | 0.19 | 0.064 | 0.047 |
| 106 | TC-445 | <0.016 | 0.032 | <0.016 | 0.032 | 0.047 | 0.125 |
| 107 | TC-448 | <0.016 | 0.023 | <0.016 | 0.016 | 0.023 | <0.016 |
| 108 | TC-454 | <0.016 | 0.016 | <0.016 | 0.023 | 0.016 | <0.016 |
| 109 | TC-459 | <0.016 | 0.012 | <0.016 | 0.023 | <0.016 | 0.19 |
| 110 | TC-481 | <0.016 | 0.032 | 0.023 | 0.125 | 0.016 | 0.047 |

# Appendix II

## The nucleotide and amino acid sequences of DNA gyrase subunit A of *H. pylori* NCTC 11637

```
atgcaagataattcagtcaatgaaacaaaaaatattgtagaagtggggattgattcttct
 M  Q  D  N  S  V  N  E  T  K  N  I  V  E  V  G  I  D  S  S
attgaagagagctatttagcttattcaatgagcgtgatcatagggcgcgccttaccggac
 I  E  E  S  Y  L  A  Y  S  M  S  V  I  I  G  R  A  L  P  D
gctagagatggcttaaagcctgtgcataggcgtattttgtatgcgatgcatgaattaggc
 A  R  D  G  L  K  P  V  H  R  R  I  L  Y  A  M  H  E  L  G
cttacttccaaagtcgcttataaaaaaagcgctaggatcgtgggtgatgtgatcggtaaa
 L  T  S  K  V  A  Y  K  K  S  A  R  I  V  G  D  V  I  G  K
taccacccccatggcgataatgcggtttatgatgcgctagtgagaatggcgcaagatttt
 Y  H  P  H  G  D  N  A  V  Y  D  A  L  V  R  M  A  Q  D  F
tctatgcgtttggaattagtggatgggcagggcaactttggctctattgatggcgataac
 S  M  R  L  E  L  V  D  G  Q  G  N  F  G  S  I  D  G  D  N
gctgcagcgatgcgttacactgaagccagaatgactaaggcgagtgaagaaattttaagg
 A  A  A  M  R  Y  T  E  A  R  M  T  K  A  S  E  E  I  L  R
gatattgataaagacaccattgattttgtgcctaattatgatgacaccttaaaagagcca
 D  I  D  K  D  T  I  D  F  V  P  N  Y  D  D  T  L  K  E  P
gatattttaacaagccgtctgcctaacctttagtcaatggggctaatgggatcgctgtg
 D  I  L  T  S  R  L  P  N  L  L  V  N  G  A  N  G  I  A  V
gggatggcgacttctatccccctcataggattgatgaaatcatagacgctttaatgcat
 G  M  A  T  S  I  P  P  H  R  I  D  E  I  I  D  A  L  M  H
gtcttagaaaaccctaacgctgaattagatgaaattttggaatttgtcaaagggcctgat
 V  L  E  N  P  N  A  E  L  D  E  I  L  E  F  V  K  G  P  D
tttcccaccggtgggatcatttatggcaaggcgggcattattgaagcctataaaacgggg
 F  P  T  G  G  I  I  Y  G  K  A  G  I  I  E  A  Y  K  T  G
cgagggcgcgtgaaagtgcgggccaaagtgcatgtggaaaagacaaaaaataaagaaatc
 R  G  R  V  K  V  R  A  K  V  H  V  E  K  T  K  N  K  E  I
atcgttttagatgaaatgcctttccaaaccaataaagccaaattagtggaacaaatcagc
 I  V  L  D  E  M  P  F  Q  T  N  K  A  K  L  V  E  Q  I  S
```

gatttagcgcgagaaaaacaaattgaaggcattagcgaagtgcgcgatgagagcgataga
D L A R E K Q I E G I S E V R D E S D R
gagggcattagagtggtgattgaattaaaaagagacgcaatgagtgaaattgtcttaaac
E G I R V V I E L K R D A M S E I V L N
catctctacaaactcaccaccatggagaccacttttagcatcattctcctcgctatttac
H L Y K L T T M E T T F S I I L L A I Y
aataaagagcctaagattttcacgcttttagagttgttgcgccttttcttaaaccacaga
N K E P K I F T L L E L L R L F L N H R
aaaaccattattataagacgcacgatttttgaattggaaaaggctaaggctagagcgcat
K T I I I R R T I F E L E K A K A R A H
attttagagggctatttgatcgcactagacaatattgatggaatcgtgcgactcattaaa
I L E G Y L I A L D N I D G I V R L I K
acaagcccaagcccagaagcggctaaaaacgccttaatggagcgtttcactttgagcgaa
T S P S P E A A K N A L M E R F T L S E
atccaaagcaaagccattttagaaatgcgtttgcaacgcttaacaggccttgaaagagac
I Q S K A I L E M R L Q R L T G L E R D
aaaatcaaagaagaataccaaaacttattagagcttattgatgatctcaatggcatttta
K I K E E Y Q N L L E L I D D L N G I L
aagagtgaagatcgcttgaatggagtcgtcaaaacagagcttttagaagtcaaagagcaa
K S E D R L N G V V K T E L L E V K E Q
ttttcttctccaaggcgcactgaaattcaagaatcctatgaaaatattgacatagaagat
F S S P R R T E I Q E S Y E N I D I E D
ttgatcgcttatgagcctatggtggtgagcatgagctataaaggctatgtgaaaagagtg
L I A Y E P M V V S M S Y K G Y V K R V
ggtttaaaagcttatgaaaagcaaaatcgtggcggtaagggcaagctttcaggcagcact
G L K A Y E K Q N R G G K G K L S G S T
tatgaagacgatttcattaaaaacttttttgtggctaacacgcatgatattttgctcttt
Y E D D F I K N F F V A N T H D I L L F
atcaccaataaggggcaattgtatcacttgaaagtctataaaatcccagaagcgagccgg
I T N K G Q L Y H L K V Y K I P E A S R
atcgctatgggtaaagccattgtgaatttaatctcgctcgctccggatgaaaagatcatg
I A M G K A I V N L I S L A P D E K I M
gcaaccctaagcaccaaagactttagcgatgaacgctctttagccttcttcacgaaaaat
A T L S T K D F S D E R S L A F F T K N
ggcgtggtgaagcgcaccaattcgagcgaatttgggaccaataggagttatagcggtatc
G V V K R T N S S E F G T N R S Y S G I

agagcgattgttttagatgaaggcgatgaattggtgagcgcaaaagttgtggataaaaac
R A I V L D E G D E L V S A K V V D K N
gctaagcatttgctcatcgcatcgcatttaggtattttcattaaattccctttagaagat
A K H L L I A S H L G I F I K F P L E D
gtgcgcgagatcggaagaaatgctcgtggggttatagggattaagctgaatgaaaatgat
V R E I G R N A R G V I G I K L N E N D
tttgttgtcggtgcggtcgtcattagcgatgatagcaacaagcttttgagcgtgagcgag
F V V G A V V I S D D S N K L L S V S E
aacgggcttggcaagcaaactctagctgaagcgtatagagagcaatctcgtggaggtaag
N G L G K Q T L A E A Y R E Q S R G G K
ggggtcattggcatgaagatcactcaaaaaaccggcaatctagtgggcgttatcagcgtg
G V I G M K I T Q K T G N L V G V I S V
gatgatgagaacctggatttgatgattcttaccgcgagcgcgaaaatgatcagagtttcc
D D E N L D L M I L T A S A K M I R V S
attaaagacattagagaaaccagaagaaatgccagtggggtaaaactcataaacaccgct
I K D I R E T R R N A S G V K L I N T A
gataaagtcgtgtatgtcaattcttgccctaaagaagaagagccagaaaatttagaaacc
D K V V Y V N S C P K E E E P E N L E T
tcttcggcgcaaaatttgtttgagtga
S S A Q N L F E -

**The nucleotide and amino acid sequences of DNA gyrase subunit B of *H. pylori* NCTC 11637**

```
atgcaaaattaccagagccatagtattaaggttttaaaaggtttagaggggttaggaaa
 M  Q  N  Y  Q  S  H  S  I  K  V  L  K  G  L  E  G  V  R  K
cgccctggaatgtatattggcgataccaatgtgggtgggttgcaccacatggtgtatgaa
 R  P  G  M  Y  I  G  D  T  N  V  G  G  L  H  H  M  V  Y  E
gtcgtggataacgctgtagatgagagcatggcgggtttttgcgatacgattaatatcact
 V  V  D  N  A  V  D  E  S  M  A  G  F  C  D  T  I  N  I  T
ttgactgatgagggttcatgcattgtagaagataacgggcgaggcattcctgtagatatt
 L  T  D  E  G  S  C  I  V  E  D  N  G  R  G  I  P  V  D  I
caccccacggaaaaaatccccgcttgcatcgtggttctaacgattttgcatgcgggaggc
 H  P  T  E  K  I  P  A  C  I  V  V  L  T  I  L  H  A  G  G
aagtttgataatgatacttataaagtttcaggcggtttgcatggcgtgggcgtttcggtt
 K  F  D  N  D  T  Y  K  V  S  G  G  L  H  G  V  G  V  S  V
gtgaacgctttgagcaaacgcttgatcatgacgattaaaaaagagggtcaaatctatcgc
 V  N  A  L  S  K  R  L  I  M  T  I  K  K  E  G  Q  I  Y  R
caagagtttgaaaagggtattcctactagcgagcttgaaatcattggcaaaaccaaaagt
 Q  E  F  E  K  G  I  P  T  S  E  L  E  I  I  G  K  T  K  S
gctaaagaaagcggcacgactattgaatttttccctgatgaaagcgtgatggaagtcgtt
 A  K  E  S  G  T  T  I  E  F  F  P  D  E  S  V  M  E  V  V
gaatttcaagcgggtattttgcaaaaacgctttaaagaaatggcgtatcttaacgacggc
 E  F  Q  A  G  I  L  Q  K  R  F  K  E  M  A  Y  L  N  D  G
ttaaaaatttctttcaaagaagaaaaaacccaactacaagagacttatttctatgaagac
 L  K  I  S  F  K  E  E  K  T  Q  L  Q  E  T  Y  F  Y  E  D
ggcttgaaacaattcgttaaagacagcgctaaaaaagaattgctcacccccattatttcg
 G  L  K  Q  F  V  K  D  S  A  K  K  E  L  L  T  P  I  I  S
tttaaaagcatggatgaagaaacgcgcacttctatagaagacgctctagcgtatgctgat
 F  K  S  M  D  E  E  T  R  T  S  I  E  D  A  L  A  Y  A  D
gattacaatgaaaacaccttaagctttgtgaataacattaaaacttctgagggcggcacg
 D  Y  N  E  N  T  L  S  F  V  N  N  I  K  T  S  E  G  G  T
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 H  E  A  G  F  K  M  G  L  S  K  A  I  L  Q  Y  I  D  N  N
attaaaaccaaagagtcgcgccccatctctgaagacattaaagagggcttgatcgctgtt
 I  K  T  K  E  S  R  P  I  S  E  D  I  K  E  G  L  I  A  V
```

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S Y A R A L V S K L V Y D K I H Q F L E
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L P G K L A D C Q S K D P L E S E I F L
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V D I E G I G K N D L M N L L K V A R H
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K E V L F E E A N Y T Y Q K L M E Y N L

```
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 I  Q  A  H  A  K  D  V  K  Q  L  D  V  -
```

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## Personalized antifungal susceptibility testing

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Sir,

In a recent issue of the Journal, Conti *et al.* concluded that so-called personalized antifungal susceptibility testing might be superior to the standardized reference method in terms of predicting the outcome of antifungal therapy.[1] They rightly point out that a wide range of factors can significantly affect the reproducibility of the test method. In this sense, antifungal agents are no different to antibacterials.

The findings of Conti *et al.* that the in-vitro activities of antifungal agents when determined in plasma are markedly different from those determined with a synthetic medium (RPMI 1640) come as no surprise, similar observations having been reported by several investigators in respect of antibacterials.[2–7] With regard to the latter, it was shown, albeit in only a few instances, that the reduced activities of these drugs in body fluids influence their clinical efficacy.[2,4] We have evaluated the activities of amphotericin B and fluconazole, alone and in combination, both in a synthetic medium (broth) and in human blood and showed that fluconazole, at certain concentrations, was fungistatic when susceptibility testing was carried out in broth, but fungicidal, at the same concentrations, when tested in blood.[8] This might explain why the results of in-vitro testing failed to predict the efficacy of fluconazole *in vivo*. For amphotericin B, we observed a marked decrease in its activity in blood which could be accounted for by the binding of the drug to the outer membranes of the cellular components of blood and the consequent reduction in the concentrations of free drug.[9,10]

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## Setting the standard for determining the in-vitro susceptibility of *Helicobacter pylori* to metronidazole

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Sir,

Metronidazole is frequently included in regimens used to eradicate *Helicobacter pylori*. However, the rates of

metronidazole resistance amongst *H. pylori* isolates are highly variable—from 11% to 70% in developed countries and even higher in developing countries.[1] As susceptibility testing of *H. pylori* has not been standardized, it may be that variations in the rates of resistance can be accounted for by differences in the methods of determining susceptibility. For example, Smith & Edwards[2] demonstrated that *H. pylori* NCTC 11637 is resistant to metronidazole when tested under microaerophilic growth conditions, but susceptible when tested under anaerobic conditions. This raises an important question: are strains which are resistant to metronidazole when in-vitro susceptibility testing is carried out under microaerophilic conditions also resistant *in vivo*? The methodology of determining the susceptibility of this bacterium to metronidazole is crucial to the ability to predict the efficacy of eradication therapy. The present study was therefore undertaken to evaluate variations in methodology on the outcome of susceptibility testing.

The MICs of metronidazole for 21 clinical isolates of *H. pylori* were determined by the Etest method (AB Biodisk, Solna, Sweden)[3] according to the manufacturer's instructions; the medium used was Columbia agar (Oxoid, Basingstoke, UK) supplemented with 7% horse blood and *H. pylori* NCTC 11637 was included as a control. The inocula were prepared by suspending colonies from 48 h growth on Columbia blood agar in Brain Heart Infusion (BHI) broth (Oxoid) and adjusting the suspensions to give turbidities equivalent to that of a No. 3 McFarland standard. The plates were incubated at 37°C in a microaerophilic atmosphere (Campylobacter System Gas Generating Kit, Oxoid) and MICs were recorded after 72 h. The majority (12) of the 21 strains, as well as the control, were resistant to metronidazole on the basis of a lower MIC breakpoint of ≥0.5 mg/L recommended by AB Biodisk; the MICs for five strains were ≥256 mg/L.

The effects of varying the conditions of susceptibility testing were evaluated with the control strain which was shown by us to be highly resistant to metronidazole (MIC ≥256 mg/L), although other investigators have reported it to be susceptible.[4,5] Firstly, the inoculated Etest plates were incubated under anaerobic conditions for 24 h before being incubated under microaerophilic conditions for a further 72 h. With the introduction of a pre-incubation phase, the MIC for *H. pylori* NCTC 11637 fell to 0.016 mg/L which is below the breakpoint defining susceptibility. This suggests that metronidazole is activated, i.e. a nitro group is reduced to a radical anion which damages DNA,[6] only under strictly anaerobic conditions. The requirement for an anaerobic atmosphere of incubation for the activation of metronidazole may explain why some centres have reported high rates of resistance to this drug amongst *H. pylori* isolates.

The next step was to compare the outcome of incubating *H. pylori* NCTC 11637 in an anaerobic jar with that in an anaerobic chamber before the plates were incubated in a microaerophilic atmosphere. Either no growth or only poor growth was observed following pre-incubation in an anaerobic chamber (Don Whitley Scientific Ltd, Shipley, UK), whereas the strain grew well when an anaerobic jar was used, suggesting that growth of *H. pylori* depends on a high level of humidity which is not provided in an anaerobic chamber.

Finally, we also investigated the effects of varying the medium, inoculum size and duration of incubation on the MICs of metronidazole for the control strain. The media evaluated were Columbia and Mueller–Hinton agar (Oxoid), both supplemented with 7% horse blood. Colonies from a 48 h culture were suspended in BHI broth and the turbidities of the suspensions were adjusted so that they were equivalent to that of either a 0.5 or a No. 3 McFarland standard. The inoculated plates were then incubated in an anaerobic atmosphere for 24 h, followed by incubation for either 48 h or 72 h in a microaerophilic atmosphere. Growth on Mueller–Hinton agar was poor, while that on Columbia agar was profuse. The MICs were difficult to interpret when the lower inoculum was used, but were clear and reproducible with the higher inoculum. After 48 h of incubation, it was not possible to record the MICs accurately because growth was too poor. On the other hand, following incubation for 72 h, the MICs were easy to read and unequivocal.

Susceptibility testing of all 21 *H. pylori* isolates to metronidazole was then repeated under optimal conditions, i.e. pre-incubation of Columbia agar plates inoculated with the higher inoculum for 24 h in an anaerobic jar, followed by incubation for 72 h in a microaerophilic atmosphere. Under these conditions, the $MIC_{50}$ and $MIC_{90}$ were 0.016 mg/L and 0.19 mg/L respectively; only one strain was truly resistant to metronidazole (MIC >256mg/L).

We have identified the optimal conditions for determining the susceptibility of *H. pylori* to metronidazole. In particular, pre-incubation under anaerobic conditions, ideally in an anaerobic jar, is regarded by us as being essential if accurate results are to be obtained. Our interpretation of the findings of this study is that *H. pylori* strains are inherently susceptible to metronidazole—a view that is in contrast with that of earlier investigators.[2] If the drug is pre-reduced, as it must be when undertaking susceptibility testing with other bacteria, then most *H. pylori* isolates will appear susceptible to it. It has been suggested previously that *H. pylori* is resistant to metronidazole when susceptibility is determined under microaerophilic conditions. We believe that, on the basis of current evidence, this conclusion is unwarranted as there is no means of confirming that the conditions that prevail during susceptibility testing are the same as those at the site of infection. None the less, the success achieved with metronidazole when it is used as treatment of patients with *H. pylori* infection suggests that most strains are susceptible to this drug, as our susceptibility test results indicate.

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## Errors associated with determining the susceptibilities of staphylococci to trimethoprim by the Vitek GPS-AK card

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Sir,

The Vitek system (bioMérieux, Hazelwood, MO, USA) was recently introduced into our laboratory as the principal means of identifying clinical isolates and performing susceptibility testing. However, concerns about the system's ability to accurately detect susceptibility to trimethoprim arose when the number of strains of methicillin-resistant *Staphylococcus aureus* (MRSA) resistant to this agent increased. Although trimethoprim is not normally regarded as first-line treatment of patients with infections caused by MRSA, it has been used successfully in combination with various other agents as oral therapy[1,2] and may also have some value as monotherapy, owing to its in-vitro activity against most staphylococci, including MRSA.[3] Trimethoprim has the added advantages of being inexpensive, being available as an oral formulation, being well absorbed and having an excellent safety profile. In common with antibiotics in general, any apparent increase in the incidence of resistance to trimethoprim will limit its potential as treatment and the investigations described here were undertaken in an attempt to identify an explanation for this phenomenon.

The susceptibilities of 100 non-replicate clinical isolates of MRSA to trimethoprim were determined in duplicate by three different methods. Vitek GPS-AK cards were inoculated, susceptibilities read according to the manufacturer's instructions and susceptibility categories assigned on the basis of MIC breakpoints recommended by the National Committee for Clinical Laboratory Standards (NCCLS), i.e. susceptible, MIC $\leq$2 mg/L and resistant, MIC $\geq$4 mg/L.[4] The Stokes' disc diffusion method was performed according to a method described previously[5] with DST agar (Oxoid, Basingstoke, UK) supplemented with 2% lysed horse blood, discs (Oxoid) containing 2.5 μg of trimethoprim and inocula with turbidities equivalent to that of a 0.5 McFarland standard. The Oxford strain of *S. aureus* was used as the control and the zones of inhibition were read after overnight incubation at 37°C. Susceptibility was defined as a zone of inhibition greater than or equal to that of the control or not >3 mm smaller and resistance as a zone of inhibition >3 mm smaller than that of the control. Finally, MICs were determined by an agar dilution method recommended by the NCCLS;[4] an MIC breakpoint of $\leq$2 mg/L was used to define susceptibility.[6]

Eighty-seven of the 100 isolates were identified as susceptible to trimethoprim by both the disc diffusion and agar dilution methods; MICs ranged from 0.25 to 1 mg/L. Of these 87, 36 (41%) were classified as susceptible and 32 (37%) as resistant with the Vitek card. Susceptibility testing of the remaining 19 strains by this method was inconclusive, the isolates being susceptible on one of the duplicate testings and resistant on the other, i.e. either 1 mg/L or 2 mg/L and $\geq$4 mg/L respectively. Thirteen isolates were found to be resistant by all three methods. Preliminary investigations with coagulase-negative staphylococci and methicillin-susceptible strains of *S. aureus* revealed patterns similar to that observed with the MRSA strains (data not shown).

In summary, the susceptibilities of 100 clinical isolates of MRSA to trimethoprim, as determined by the disc diffusion and agar dilution methods, were identical, with 87 strains being categorized as susceptible. The Vitek GPS-AK card, on the other hand, incorrectly designated 32 of these 87 strains as resistant—a major error rate of 37%. Moreover, the results of susceptibility testing of a further 19 strains were not reproducible and, therefore, inconclusive. These observations suggest that the Vitek GPS-AK card is an unreliable method of determining the susceptibilities of staphylococci to trimethoprim.

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# High-Level Fluoroquinolone Resistance in Helicobacter pylori Caused by Three Unusual Sequential Mutations in gyrA

Ciprofloxacin resistance in Helicobacter pylori is due to the alteration within the gyrA gene, encoding the A subunit of DNA gyrase. To investigate the development of resistance in H. pylori, ciprofloxacin-resistant mutants were selected in vitro by exposing a sensitive strain to serial increments of ciprofloxacin in Columbia blood agar plate. The Quinolone Resistance-Determining Region (QRDR) of gyrA gene was analysed for mutations. Gene amplification was performed by PCR and the resultant fragments were sequenced. Reduced susceptibility (MIC 8-128 mg/L) to ciprofloxacin was associated with either a single or double amino acid changes in gyrA gene. The first-step mutant (MIC 8 mg/L) carried one gyrA mutation at amino acid 91 (Asp to Asn). The second-step mutant (MIC 64 mg/L) had an additional gyrA mutation at amino acid 85 (Gly to Cys) and the third-step mutant (MIC 128 mg/L) showed the mutation at amino acid 91 (Asn to Tyr). A comparative in vivo study for ciprofloxacin resistance was determined by a clinical ciprofloxacin resistant isolate with the MIC 32 mg/L. This strain had a single amino acid substitution at codon 87 (Asn to Lys). After the in vitro selection mutation, no further alteration in the QRDR was observed when the MIC was increased to 64 mg/L. This latter observation demonstrates that gyrA mutations are not sole contributors for the mechanism of ciprofloxacin resistance in H. pylori. As no parC gene has yet been identified in H. pylori, any contribution from topoisomerase IV mutants cannot be quantified. High level resistance to ciprofloxacin was associated with two mutation sites in the gyrA gene in laboratory mutant. Although a single mutation at Asn-87, which is homologous to Ser-83 in E. coli, has been linked to high level resistance to ciprofloxacin in H. pylori. In this study the mutations at amino acid 91 may be comparable with the codon 87 mutation in E. coli gyrA but no direct comparison can be made with mutations in H. pylori codon 85.

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# Fluoroquinolone Resistance in *Helicobacter pylori*: Novel Sequential *gyrA* Mutation Combinations

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## Abstract

Fluoroquinolone resistance in *Helicobacter pylori* has been related to point mutations in the Quinolone Resistance-Determining Region (QRDR) of the A subunit of DNA gyrase, encoded by the *gyrA* gene. We have previously demonstrated that either a single mutation at Asn87 or double amino acid changes at Asp91 and Gly85 in the *gyrA* gene are associated with high-level ciprofloxacin resistance. In this study, we characterised the sequential mutations in *gyrA* in two different laboratory mutants. Ciprofloxacin-resistant mutants were obtained from *H. pylori* NCTC 11916 and HP-7 by stepwise selection on increasing concentrations of ciprofloxacin. The QRDR of *gyrA* in resistant mutants was amplified by PCR and sequenced. The two first-step mutants (both with an MIC of 16 mg/L) had the same *gyrA* mutation at Asp91 to Asn. One of the second-step mutants (from HP-7) had the MIC of 128 mg/L accompanied by an additional mutation at Asn87 to Lys. A second-step mutation of NCTC 11916 raised the MIC to 64 mg/L but no change was found in the sequence. On the other hand, the third step mutation of NCTC 11916 had an MIC of 128 mg/L and now carried the additional mutation at Asn87 to Lys. The third-step mutation of HP-7 (MIC 512 mg/L) showed no change in sequence. This is the first report showing double amino acid changes at Asn87 to Lys and Asp91 to Asn in *H. pylori*, albeit that they may manifest themselves at different mutation stages. These results suggest that, in addition to *gyrA* mutations, other mechanisms of resistance may play a role in the mechanisms of ciprofloxacin resistance in *H. pylori* since no additional mutation was identified in the second-step mutation of NCTC 11916 and the third-step mutation of HP-7. Mutations in a *parC* gene, which has not yet been identified in *H. pylori*, are thought unlikely to make any contributions toward fluoroquinolone resistance.

## Introduction

Fluoroquinolones have a broad antibacterial spectrum against gram negative and gram positive bacteria and exert their antibacterial actions by inhibiting type II topoisomerases- DNA gyrase and Topoisomerase IV. In *Helicobacter pylori*, fluoroquinolone resistance appears to be due to mutation in the Quinolone-Resistance-Determining Region (QRDR) of the GyrA subunit of DNA gyrase[1]. Our previous study has demonstrated that either a single mutation at Asn87 or double amino acid changes at Asp91 and Gly85 in GyrA confer high-level ciprofloxacin resistance[2]. In this study, we characterised the sequential mutations in the QRDR of the *gyrA* gene of two different laboratory ciprofloxacin-resistant mutants to investigate the development of ciprofloxacin resistance in *H. pylori*.

## Methods

Stepwise selection of ciprofloxacin-resistant *H. pylori* mutants

Ciprofloxacin-sensitive *H. pylori* NCTC 11916 and HP-7 were used to select ciprofloxacin-resistant mutants. It was performed by plating approximately $10^8$ cfu of *H. pylori* onto Columbia blood agar containing increasing concentrations of ciprofloxacin. After 72 hours of incubation at 37°C under microaerophilic conditions, first-step mutants were selected. Second-step mutants were obtained by plating the first-step mutants onto Columbia blood agar containing increasing concentrations of ciprofloxacin.Third-step mutants were obtained in the similar manner to the second-step mutants.

Amplification of the QRDR of the *gyrA* gene and DNA sequencing

Primers and PCR amplification conditions were carried out as described by Moore et al[1]. The DNA sequencing was done by an automatic sequencer.

## Results

The MICs and sequential mutations in GyrA are shown in Table1 and Figure 1. The two first-step mutants (HP7.1 and NCTC 11916A) had the same *gyrA* mutation at Asp91 to Asn. The second-step mutants HP-7.2 (from HP-7) had the MIC of 128 mg/L and carried an additional mutation at Asn87 to Lys. A second-step mutation of NCTC 11916 (NCTC 11916B) raised the MIC to 64 mg/L but no change was found in the sequence. On the other hand, the third step mutation of NCTC 11916 (NCTC 11916C, MIC 128 mg/L) now had the additional mutation at Asn87 to Lys. The third-step mutation of HP-7 (HP-7.3, MIC 512 mg/L) had no change in sequence. These results suggest that other mechanisms of resistance may play a role in the mechanisms of ciprofloxacin resistance in *H. pylori* since no additional mutation was identified in the second-step mutation of NCTC 11916 and the third-step mutation of HP-7.

Table 1 Laboratory-induced mutations within the QRDR of *H. pylori* GyrA

| Strain | MIC (mg/L) | GyrA mutations |
|---|---|---|
| HP-7 | 0.032 | - |
| NCTC 11916 | 0.064 | - |
| 1st step | | |
| HP-7.1 | 16 | Asp91 → Asn |
| NCTC 11916A | 16 | Asp91 → Asn |
| 2nd step | | |
| HP-7.2 | 128 | Asp91 → Asn, Asn87 → Lys |
| NCTC 11916B | 64 | Asp91 → Asn |
| 3rd step | | |
| HP-7.3 | 512 | Asp91 → Asn, Asn87 → Lys |
| NCTC 11916C | 128 | Asp91 → Asn, Asn87 → Lys |

Figure 1 Sequential mutations in the QRDR of GyrA in laboratory mutants

| | 87 91 | |
|---|---|---|
| **HP-7** | **VIGKYHPHGDNAVYDALVRMAQDFSM** | **(MIC 0.032 mg/L)** |
| **NCTC 11916** | **VIGKYHPHGDNAVYDALVRMAQDFSM** | **(MIC 0.064 mg/L)** |
| Laboratory mutants of HP-7 | | |
| | 87 91 | |
| 1st step mutant | **VIGKYHPHGDNAVYNALVRMAQDFSM** | **(MIC 16 mg/L)** |
| 2nd step mutant | **VIGKYHPHGDKAVYNALVRMAQDFSM** | **(MIC 128 mg/L)** |
| 3rd step mutant | **VIGKYHPHGDKAVYNALVRMAQDFSM** | **(MIC 512 mg/L)** |
| Laboratory mutants of NCTC 11916 | | |
| | 87 91 | |
| 1st step mutant | **VIGKYHPHGDNAVYNALVRMAQDFSM** | **(MIC 16 mg/L)** |
| 2nd step mutant | **VIGKYHPHGDNAVYNALVRMAQDFSM** | **(MIC 64 mg/L)** |
| 3rd step mutant | **VIGKYHPHGDKAVYNALVRMAQDFSM** | **(MIC 128 mg/L)** |

**D = Aspartic acid** K = Lysine N = Asparagine

## Conclusions

- High-level ciprofloxacin resistance in *H. pylori was* associated with two mutation sites in the *gyrA* gene in laboratory mutants.
- This is the first report showing double amino acid changes at Asn87 to Lys and Asp91 to Asn in *H. pylori*, albeit that they may manifest themselves at different mutation stages.
- In addition to *gyrA* mutations, other mechanisms of resistance may play a role in the mechanisms of ciprofloxacin resistance in *H. pylori*.
- A *parC* gene has not yet been identified in *H. pylori*, therefore, mutations in topoisomerase IV may not contribute to the mechanism of fluoroquinolone resistance.

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# Antimicrobial Susceptibility Patterns of *Helicobacter pylori* Isolated in Scotland

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## Abstract

*Helicobacter pylori* is the principal cause of chronic active gastritis and peptic ulcer disease. Multi-drug antimicrobial therapies include combinations of proton pump inhibitors and one or several antimicrobial agents. Resistance of *H. pylori* to antimicrobial agents has been associated with treatment failure. Between December 1999 and August 2000, we obtained 243 antral biopsies from patients undergoing endoscopy in Edinburgh. A total of 67 *H. pylori* strains were isolated (27.6%). Antimicrobial susceptibility patterns of amoxycillin (AMX), ciprofloxacin (CIP), clarithromycin (CLA), erythromycin (ERY), tetracycline (TET) and metronidazole (MTZ) were determined by E test. Mutations in the 23S rRNA conferring macrolide resistance were investigated by PCR and DNA sequencing. $MIC_{50}$ and $MIC_{90}$ values are shown below. Resistance to clarithromycin and erythromycin was found in 12% (8/67) and 13% (9/67) of the *H. pylori* strains, respectively. All clarithromycin-resistant isolates had erythromycin MIC of >256 mg/L. Metronidazole resistance was demonstrated in 6 isolates (9%). Two of them had clarithromycin and erythromycin resistance. Tetracycline resistance (intermediate) was found in one of the 67 isolates (1.5%). Resistance to amoxycillin and ciprofloxacin was not detected.

| Antimicrobial agent | $MIC_{50}$ (mg/L) | $MIC_{90}$ (mg/L) |
|---|---|---|
| Amoxycillin | <0.016 | 0.016 |
| Ciprofloxacin | 0.032 | 0.064 |
| Clarithromycin | <0.016 | 3 |
| Erythromycin | 0.023 | >256 |
| Tetracycline | 0.023 | 0.064 |
| Metronidazole | <0.016 | 0.75 |

Mutations in the 23S rRNA gene were examined in the nine macrolide-resistant *H. pylori* strains. Seven of the nine isolates had mutation at position 2143 (A to G). Two of the seven isolates had an additional T to C mutation at either position 2182 or 1934. Mutation at position 2182 (T to C) has previously been found not to be associated with macrolide resistance. However, the mutation at position 1934 has not previously been reported. Of the nine isolates, one carried only a T to C mutation at position 2182 and one had no mutation in the 23S rRNA, implying that other mechanisms are responsible for the resistance in these strains. The results show that macrolide resistance, occurring by different mechanisms, is currently found in 13% of our *H. pylori* isolates.

## Introduction

*Helicobacter pylori* plays an important role in chronic active gastritis and peptic ulcer disease.[1] Multi-drug antimicrobial therapies including combinations of clarithromycin, metronidazole or amoxycillin and proton pump inhibitors are widely used to eradicate *H. pylori*.[2] The development of antimicrobial resistance in *H. pylori* has been associated with treatment failure. Mutations in the peptidyltransferase region of the 23S rRNA of *H. pylori* confer macrolide resistance.[3] In this study, we investigated the antimicrobial susceptibility patterns of amoxycillin, ciprofloxacin, clarithromycin, erythromycin, tetracycline and metronidazole in *H. pylori* strains isolated in Edinburgh, Scotland. Mutations in the 23S rRNA gene conferring macrolide resistance were also evaluated.

## Methods

### Bacterial strains

A total of 67 *H. pylori* strains isolated from 243 antral biopsy samples of patients undergoing endoscopy at the Centre for Liver and Digestive Disorders, Royal Infirmary, Edinburgh, Scotland. The samples were collected during the period December 1999 - August 2000.

### Susceptibility testing

MICs of amoxycillin, ciprofloxacin, clarithromycin, erythromycin, tetracycline and metronidazole were determined by E test with Columbia agar supplemented with 7% horse blood.

### PCR amplification and sequencing of the 23S rRNA gene

Primers and PCR amplification conditions were carried out as described by Occhialini et al.[4] The DNA sequencing was determined by an automatic sequencer.

## Results

The $MIC_{50}$ and $MIC_{90}$ of the six antibiotics are shown in Table 1. Resistant to clarithromycin and erythromycin was found in 12% (8/67) and 13% (9/67) of the *H. pylori* strains, respectively. All of these strains had erythromycin MIC of >256 mg/L. Six isolates were resistant to metronidazole and two of them also had clarithromycin and erythromycin resistance. Tetracycline resistance (intermediate, MIC 1 mg/L) was found in one of the 67 isolates (1.5%). Resistance to amoxycillin and ciprofloxacin was not detected.

Mutations in the 23S rRNA of 9 macrolide-resistant isolates are presented in Table 2. The A to G mutations at position 2143 were identified in 7 isolates. Two of them had an additional mutation from T to C at either position 1934 or 2182. Mutation at position 2182 (T to C) has previously been found not to be associated with macrolide resistance.[5] However, the mutation at position 1934 has not previously been reported. Of the nine isolates, one carried only a T to C mutation at position 2182 and one had no mutation in the 23S rRNA , implying that other mechanisms are responsible for the resistance in these strains. The results show that macrolide resistance, occurring by different mechanisms, is currently found in 13% of our *H. pylori* isolates.

Table 1. Antimicrobial susceptibility patterns of 67 *H. pylori* clinical isolates

| Antimicrobial agent | $MIC_{50}$ (mg/L) | $MIC_{90}$ (mg/L) | Number of isolates | | |
|---|---|---|---|---|---|
| | | | resistant | intermediate | susceptible |
| Amoxycillin | <0.016 | 0.016 | 0 | 0 | 67 |
| Ciprofloxacin | 0.032 | 0.064 | 0 | 0 | 67 |
| Clarithromycin | <0.016 | 3 | 7 | 1 | 59 |
| Erythromycin | 0.023 | >256 | 9 | 0 | 58 |
| Tetracycline | 0.023 | 0.064 | 0 | 1 | 66 |
| Metronidazole | <0.016 | 0.75 | 6 | 0 | 61 |

Table 2. Mutations in the 23S rRNA gene of 9 macrolide-resistant *H. pylori*

| Strain No. | MIC(mg/L) | | 23S rRNA mutations |
|---|---|---|---|
| | Clarithromycin | Erythromycin | |
| TC-18 | >256 | >256 | - |
| TC-24 | >256 | >256 | A2143G, T2182C |
| TC-40 | 32 | >256 | A2143G, T1934C |
| TC-78 | 6 | >256 | A2143G |
| TC-19 | 1.5 | >256 | A2143G |
| TC-140 | 3 | >256 | A2143G |
| TC-175 | 0.125 | >256 | T2182C |
| TC-185 | 24 | >256 | A2143G |
| TC-215 | 3 | >256 | A2143G |

## Conclusions

- The prevalence of macrolide resistance (13%) is greater than other antibiotics.
- Clarithromycin resistance in *H. pylori* in Scotland has emerged. Resistance to clarithromycin will affect the cure rates of *H. pylori* infection and account for treatment failure.
- Mutations in the peptidyltransferase region of the 23S rRNA in *H. pylori* are associated with macrolide resistance.

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# Antimicrobial Susceptibilities and Molecular Mechanism of Macrolide and Fluoroquinolone Resistances in *Helicobacter pylori* Isolated in Scotland

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## Abstract

Antimicrobial resistance in *Helicobacter pylori* has been associated with treatment failure. Between December 1999 and February 2001, a total of 100 *H. pylori* strains (24%) were isolated from 418 antral biopsy samples of patients undergoing endoscopy in Edinburgh. Antimicrobial susceptibility patterns of amoxycillin, ciprofloxacin, clarithromycin, erythromycin, tetracycline and metronidazole were determined by E test. Mutations in the 23S rRNA and the Quinolone Resistant-Determining Region (QRDR) of *gyrA* gene were investigated for the mechanisms of macrolide and fluoroquinolone resistances, respectively. $MIC_{50}$ and $MIC_{90}$ values of six antibiotics are shown below. Resistance to clarithromycin and erythromycin was found in 9% and 10% of the *H. pylori* strains, respectively. All clarithromycin-resistant isolates had erythromycin MIC of >256 mg/L. Metronidazole resistance was found in 8 isolates (8%). Two of them had clarithromycin and erythromycin resistance. Ciprofloxacin resistance was demonstrated in 2 isolates (2%). Tetracycline resistance (intermediate, MIC 1 mg/L) was found in one isolate (1%). Resistance to amoxycillin was not detected.

| Antimicrobial agent | $MIC_{50}$ (mg/L) | $MIC_{90}$ (mg/L) |
|---|---|---|
| Amoxycillin | <0.016 | 0.016 |
| Ciprofloxacin | 0.032 | 0.064 |
| Clarithromycin | <0.016 | 0.047 |
| Erythromycin | 0.023 | 0.125 |
| Tetracycline | 0.023 | 0.064 |
| Metronidazole | 0.016 | 1 |

Fluoroquinolone resistance was associated with alterations in the QRDR of *gyrA* gene. Mutation at position 91 (Asp to Asn) was found in both ciprofloxacin-resistant strains (MIC 16 and 32 mg/L). Seven of the ten macrolide-resistant isolates had mutations in the 23S rRNA at position 2143 (A to G). Two of the seven isolates had an additional T to C mutation at either position 2182 or 1934. Of the ten isolates, two carried a single mutation at either position 2182 or 2195. Mutation at position 2182 has previously been found not to be associated with macrolide resistance. However, the mutations at position at 1934 (T to C) and position 2195 (C to T) have not previously been identified. No mutation was detected in one isolate. The results imply that different mechanisms are responsible for macrolide resistance in our *H. pylori* strains.

## Introduction

Multiple antimicrobial therapies including combinations of clarithromycin, metronidazole or amoxycillin and proton pump inhibitors are widely used to eradicate *H. pylori*. However, antimicrobial resistance in *H. pylori* has emerged and is considered as the principal cause of treatment failure. In this study, we investigated the antimicrobial susceptibility patterns of amoxycillin, ciprofloxacin, clarithromycin, erythromycin, tetracycline and metronidazole in *H. pylori* strains isolated in Edinburgh, Scotland. Mutations within the peptidyltransferase region of the 23S rRNA and mutations in the QRDR of *gyrA* gene were also evaluated

## Methods

### Bacterial strains

A total of 100 *H. pylori* strains were isolated from 418 antral biopsies of patients undergoing endoscopy at the Centre for Liver and Digestive Disorders, Royal Infirmary, Edinburgh, Scotland. The specimens were collected during the period December 1999 - February 2001.

### Susceptibility testing

MICs of amoxycillin, ciprofloxacin, clarithromycin, erythromycin, tetracycline and metronidazole were determined by E test.

### Mechanisms of macrolide and fluoroquinolone resistances

The peptidyltranferase region of 23S rRNA gene and the QRDR of *gyrA* gene were amplified and sequenced in macrolide-resistant and ciprofloxacin-resistant strains, respectively[1,2].

## Results

The $MIC_{50}$ and $MIC_{90}$ of the six antibiotics are shown in Table 1. Fluoroquinolone resistance was demonstrated in 2 isolates. Mutation at position 91 (Asp to Asn) of the *gyrA* gene was detected in both ciprofloxacin-resistant strains (Figure 1).

Resistance to clarithromycin and erythromycin was found in 9 and 10 isolates, respectively. Mutations in the 23S rRNA of macrolide-resistant strains are demonstrated in Table 2. All clarithromycin-resistant isolates had erythromycin MIC of >256 mg/L. Seven of the ten macrolide-resistant isolates had mutations in the 23S rRNA at position 2143 (A to G). Two of the seven isolates had an additional T to C mutation at either position 2182 or 1934. Of the ten isolates, two carried a single mutation at either position 2182 or 2195. Mutation at position 2182 has previously been found not to be associated with macrolide resistance. However, the mutations at position at 1934 (T to C) and position 2195 (C to T) have not previously been identified. No mutation was detected in one isolate.

Metronidazole resistance was demonstrated in 8 isolates (8%). Two of them had clarithromycin and erythromycin resistance. Tetracycline resistance (intermediate, MIC 1 mg/L) was found in one isolate (1%). Resistance to amoxycillin was not detected.

Table 1 Antimicrobial susceptibility patterns of 100 *H. pylori* isolates

| Antimicrobial Agent | $MIC_{50}$ | $MIC_{90}$ |
|---|---|---|
| Amoxycillin | <0.016 | 0.016 |
| Ciprofloxacin | 0.032 | 0.064 |
| Clarithromycin | <0.016 | 0.047 |
| Erythromycin | 0.023 | 0.125 |
| Tetracycline | 0.023 | 0.064 |

Table 2 Mutations in the 23S rRNA gene of 10 macrolide-resistant *H. pylori*

| Strain | MIC (mg/L) | | 23S rRNA mutations |
|---|---|---|---|
| | Clarithromycin | Erythromycin | |
| TC-18 | >256 | >256 | - |
| TC-24 | >256 | >256 | A2143G, T2182C |
| TC-40 | 32 | >256 | A2143G, T1934C |
| TC-78 | 6 | >256 | A2143G |
| TC-19 | 1.5 | >256 | A2143G |
| TC-140 | 3 | >256 | A2143G |
| TC-175 | 0.125 | >256 | T2182C |
| TC-185 | 24 | >256 | A2143G |
| TC-215 | 3 | >256 | A2143G |
| TC-284 | 8 | >256 | C2195T |

Figure 1 Mutations in the QRDR of *gyrA* gene in ciprofloxacin-resistant isolates

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                          91
NCTC 11916 VIGKYHPHGDNAVYDALVRMAQDFSM (MIC 0.064 mg/L)
TC-246     VIGKYHPHGDNAVYNALVRMAQDFSM (MIC 16 mg/L)
TC-413     VIGKYHPHGDNAVYNALVRMAQDFSM (MIC >32 mg/L)

D = Aspartic acid    N = Asparagine
```

## Conclusions

- The prevalence of macrolide resistance (10%) is greater than other antibiotics.
- Mutations in the peptidyltransferase region of the 23S rRNA in *H. pylori* are associated with macrolide resistance.
- Fluoroquinolone resistance is associated with mutations in the QRDR of *gyrA* gene.

### References

1. Occhialini, A., M. Urdaci, F. Daucet-Populaire, C. M.Bebear, H. Lamouliatte, and F. Megraud. 1997. Macrolide resistance in *Helicobacter pylori*: rapid detection of point mutations and assays of macrolide binding to ribosomes. Antimicrob. Agent Chemother. 41:2724-28

2. Moore RA, Beckthold B, Wong S, Kureishi A, and Bryan EL. 1995. Nucleotide sequence of the *gyrA* gene and characterization of ciprofloxacin-resistant mutants of *Helicobacter pylori*. Antimicrob. Agents Chemothers. 39: 107-111.

# Antimicrobial Resistance of *Helicobacter pylori* Strains Isolated in Scotland

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## Introduction

*Helicobacter pylori* plays an important role in chronic active gastritis and peptic ulcer disease. Multiple antimicrobial therapies including combinations of clarithromycin, metronidazole or amoxycillin and proton pump inhibitors are widely used to eradicate *H. pylori*. However, antimicrobial resistance in *H. pylori* has emerged and is considered as the principal cause of treatment failure.

## Aims

- To determine antibiotic susceptibility patterns of *H. pylori* strains isolated in Scotland.
- To investigate mutations within the peptidyltransferase region of the 23S rRNA gene and mutations in the QRDR of the *gyrA* gene in macrolide-resistant and ciprofloxacin-resistant isolates, respectively.

## Methods

### Bacterial strains

A total of 110 *H. pylori* strains were isolated from 454 antral biopsies of patients undergoing endoscopy at the Centre for Liver and Digestive Disorders, Royal Infirmary, Edinburgh, Scotland. The specimens were collected during the period December 1999 - March 2001.

### Susceptibility testing

The MICs of amoxycillin, ciprofloxacin, clarithromycin, erythromycin, tetracycline and metronidazole were determined by E test with Columbia agar supplemented with 7% horse blood.

### Mechanisms of macrolide and fluoroquinolone resistance

The peptidyltranferase region of the 23S rRNA gene and the QRDR of the *gyrA* gene were amplified and sequenced in macrolide-resistant and ciprofloxacin-resistant strains, respectively[1,2].

Table 1 Antimicrobial susceptibility patterns of 110 *H. pylori* isolates

| Antimicrobial Agent | MIC (mg/L) Range | $MIC_{50}$ | $MIC_{90}$ | % Resistance |
|---|---|---|---|---|
| Amoxycillin | <0.016-0.047 | <0.016 | 0.016 | 0 |
| Ciprofloxacin | 0.003->32 | 0.032 | 0.064 | 1.8 |
| Clarithromycin | <0.016->256 | <0.016 | 0.023 | 8.2 |
| Erythromycin | <0.016->256 | 0.023 | 0.125 | 9.1 |
| Tetracycline | <0.016-1 | 0.023 | 0.064 | 0.9 |
| Metronidazole | <0.016->256 | 0.016 | 0.75 | 7.3 |

Figure 1 Mutations in the QRDR of *gyrA* in ciprofloxacin-resistant isolates

```
                      91
NCTC 11916 VIGKYHPHGDNAVYDALVRMAQDFSM (MIC 0.064 mg/L)
TC-246     VIGKYHPHGDNAVYNALVRMAQDFSM (MIC 16 mg/L)
TC-413     VIGKYHPHGDNAVYNALVRMAQDFSM (MIC >32 mg/L)

D = Aspartic acid    N = Asparagine
```

Table 2 Mutations in the 23S rRNA gene of 10 macrolide-resistant *H. pylori*

| Strain | MIC (mg/L) Clarithromycin | Erythromycin | 23S rRNA mutations |
|---|---|---|---|
| TC-18 | >256 | >256 | - |
| TC-24 | >256 | >256 | A2143G, T2182C |
| TC-40 | 32 | >256 | A2143G, T1934C |
| TC-78 | 6 | >256 | A2143G |
| TC-19 | 1.5 | >256 | A2143G |
| TC-140 | 3 | >256 | A2143G |
| TC-175 | 0.125 | >256 | T2182C |
| TC-185 | 24 | >256 | A2143G |
| TC-215 | 3 | >256 | A2143G |
| TC-284 | 8 | >256 | C2195T |

## Results

The $MIC_s$ of the six antibiotics are shown in Table 1. Fluoroquinolone resistance was demonstrated in 2 isolates (1.8%). Mutation at position 91 (Asp to Asn) of the *gyrA* gene was detected in both ciprofloxacin-resistant strains (Figure 1).

Resistance to clarithromycin and erythromycin was found in 8.2% (9/110) and 9.1% (10/110) of the *H. pylori* isolates, respectively. Mutations in the 23S rRNA of macrolide-resistant strains are demonstrated in Table 2. Seven of the ten macrolide-resistant isolates had mutations in the 23S rRNA at position 2143 (A to G). Two of the seven isolates had an additional T to C mutation at either position 2182 or 1934. Of the ten isolates, two carried a single mutation at either position 2182 or 2195. Mutation at position 2182 has previously been found not to be associated with macrolide resistance. However, the mutations at position at 1934 (T to C) and position 2195 (C to T) have not previously been identified. No mutation was detected in one isolate. Metronidazole resistance was demonstrated in 8 isolates (7.3%). Two of them had clarithromycin and erythromycin resistance. Tetracycline resistance (intermediate, MIC 1mg/L) was found in one isolate (0.9%). Resistance to amoxycillin was not detected.

## Conclusions

- The prevalence of macrolide resistance (9.1%) is greater than other antibiotics.
- Mutations in the peptidyltransferase region of the 23S rRNA gene in *H. pylori* are associated with macrolide resistance.
- Fluoroquinolone resistance is associated with mutations in the QRDR of the *gyrA* gene.

### References

1. Occhialini, A., M. Urdaci, F. Daucet-Populaire, C. M.Bebear, H. Lamouliatte, and F. Megraud. 1997. Macrolide resistance in *Helicobacter pylori*: rapid detection of point mutations and assays of macrolide binding to ribosomes. Antimicrob. Agents Chemother. 41:2724-28

2. Moore RA, Beckthold B, Wong S, Kureishi A, and Bryan EL. 1995. Nucleotide sequence of the *gyrA* gene and characterization of ciprofloxacin-resistant mutants of *Helicobacter pylori*. Antimicrob. Agents Chemother. 39: 107-111.