# Fluoroquinolone Action and Resistance: Action of Ciprofloxacin in a Standard *Escherichia coli* and the Roles of *GyrA* and *ParC* Mutations in High-Level Resistance

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# ABSTRACT

DNA gyrase is a lethal bacterial enzyme that is a target of the fluoroquinolones, one of the best antibiotic classes used in chemotherapy, the only reliable postinfection option to fight against bacterial infections. However, emergence of resistance has been being found inevitably leading to more difficulty in the treatment of infections caused by higher resistance each year. It is therefore important to study further how some clinical bacteria develop their resistance, especially those with very high resistance, in order to establish more effective and appropriate ways of preventing bacterial resistance. Several bacterial isolates from Malaysia and Thailand were found and tested for their MIC of ciprofloxacin (Cip). Some had very to extremely high resistance including three *Acinetobacter baumanii* (MIC of 128 mg/L). *GyrA* and *parC* are homologous genes and are subunits of enzyme gyrase and topoisomerase IV, respectively. By using the whole cell-directed PCR technique, magnetic biotin-streptavidin separation (Dynabeads®M-280 Streptavidin) and direct DNA sequencing, sequence analysis showed that: three *Escherichia coli* (MIC of 128 mg/L) had two mutations in *gyrA* (Ser-83-Leu and Asp-87-Tyr) and one mutation in *parC* (Ser-83-Ile). Another *E. coli* (MIC of 32 mg/L) had the same three mutations except at *gyrA* 87 the mutation was Asp-87-Asn. Thus Tyr substitution seems to enhance resistance more than any other mutations. One *Citrobacter freundii* (MIC of 128 mg/L) had Thr-83-Ile and Asp-87-Tyr; and no mutation in *parC*'s QRDR. *Enterobacter sakazakii* (MIC of 32 mg/L) had two mutations in *gyrA's* QRDR: Ser-

83-Tyr and Asp-87-Asn, and one mutation in *parC*'s` QRDR: Ser-83-Ile. Moreover, one *Moraxella catarrhalis* (found in Edinburgh) with high Cip resistant at MIC of 4.0 mg/L had one mutation in *gyrA*'s QRDR: Gly-87-Asn, and its *parC*'s QRDR was not to be revealed. Mutations in both *gyrA*'s and *parC*'s QRDR at amino acid residues 83 and 87 are probably the main mechanism of Cip resistance of those isolates tested. (Amino acid positions appeared above are these equivalent to *E. coli*'s *gyrA*.) *gyrA's* and *parC*'s QRDR of *Citrobacter freundii* and *Enterobacter sakazakii*, and also *gyrA's* QRDR of *Moraxella catarrhalis* were revealed perhaps for the first time. Unique *gyrA* silent-nucleotide alterations of eight *E. coli* from Malaysia and Thailand were found to be common to both sensitive and resistant isolates at position 438, 456, 483 and 516, which differed from all *E. coli* published sequences in GENBANK.

Mutual antagonism between chloramphenicol (Cm) and rifampicin (Rif) against Cip was examined on *E. coli* NCTC10418. In response to Cip, *E. coli* KL16 showed a convex curve, not the commonly found biphasic. Cip bactericidal activity was much reduced when the *E. coli* was exposed to Cm, regardless of how long (0 to 120 min) the bacteria were subjected to Cm. Cip regained its strongly bactericidal effect on the cells after Cm had been removed. Rif did not antagonise Cip activity when the cells were challenged with both Rif and Cip at the same time after a previous 30-min exposure of the cells to Rif.

# DECLARATION

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

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For their unending love and support,

I dedicate this thesis to my parents

and grandmother.

# ABBREVIATIONS

| | |
|---|---|
| bp | Base pair |
| BSA | Bovine serum albumin |
| cfu | Colony forming units |
| CLED medium | Cystine-lactose-electrolyte deficient medium |
| Cm | Chloramphenicol |
| Cip | Ciprofloxacin |
| dATP | Deoxy adenosine 5'-triphosphate |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxyribonucleoside-5'-triphosphate |
| DTT | Dithiothreitol |
| EDTA | Ethylenediaminetetraacetic acid |
| Eno | Enoxacin |
| ID50 | Dose giving 50% inhibition |
| kb | Kilobases |
| kbp | Kilobase pairs |
| Kda | Kilo Daltons |
| log | Logrithm |
| MIC | Minimum inhibitory concentration |
| min | Minutes |
| NA | Nalidixic acid |
| Nfx | Norfloxacin |
| O.D. | Optical density |

| | |
|---|---|
| Ofx | Ofloxacin |
| PAGE | Polyacrylamide gel electrophoresis |
| QRDR | Quinolone resistant determining region |
| Rif | Rifampicin |
| RNA | Ribonucleic acid |
| SDS | Sodium dodecyl sulphate (sodium lauryl sulphate) |
| TEMED | N,N,N',N'-tetramethyl-ethylenediamine |
| Tris | Tris (hydroxymethyl) methylamide |

The standard single and three letter abbreviations are used for the amino acids.

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# 1. INTRODUCTION

Fluoroquinolones are highly potent members of a class of antibacterial agents called quinolones, new totally synthetic broad-spectrum antibacterial agents developed for treatment of infections in humans ranging from urinary tract infections to life-threatening septicaemia. It is the drugs's remarkable potency and novel mode of action against many bacterial species that leads to an optimistic view of the potential for resistance development. Nine to 13 years of clinical use in almost every country in the world demonstrates the universal acceptance of these drugs, however it also raises concerns about the development and incidence of resistant strains inevitably leading to difficulty in the treatment of infections.

In the fight against bacterial diseases, antibiotic therapy is the only reliable postinfection option. However, the use of antibacterial therapy has almost always led to the development of bacterial resistance. Fortunately, resistance to 4-quinolones is still rare in common pathogens with 97 to 100% of strains remaining susceptible (Acar *et. al.*, 1993). Resistance has been reported in methicillin-susceptible *Staphylococcus aureus*, *Campylobacter jejuni/coli*, *Salmonella* spp., *Shigella* spp. and *Escherichia coli* (Acar *et. al.*, 1993). The highest incidence of resistance is observed in *Serratia* and *Acinetobacter* spp., and particularly in methicillin-resistant *S. aureus* (Acar *et. al.*, 1993).

Wide-scale resistance to 4-quinolones has developed in both non- and pathogens with the wide and careless use of quinolones; resistance is now a problem and is set to become a major problem soon. In order to reduce the inevitable emergence of 4-

quinolone resistance, treatment with these drugs should be carefully controlled. It is imperative that we use these drugs to their maximum potential so that the development of resistance is minimized. This means that further studies should be performed to establish more effective and appropriate ways of preventing bacterial resistance. The purpose of this thesis is to understand further the action of the fluoroquinolones, the mechanisms involved in the development of resistance and then to devise strategies to use these drugs more effectively.

## QUINOLONES

4-Quinolones are synthetic antibacterial drugs whose chemical structures are never found in nature. They are a new class of totally synthetic broad-spectrum antibacterial agents. They are not strictly-speaking antibiotics but they are now usually referred to as such. On the basis of their structures and antimicrobial spectra, quinolones can be separated into two groups (Chu & Shen, 1995): first-generation quinolones and newer second-generation quinolones.

1. **First-generation quinolones** include nalidixic acid, oxolinic acid, pipemidic acid, piromidic acid and cinoxacin. Nalidixic acid, discovered in 1962, was the first member of the antibacterial quinolones and was found to be active against Gram-negative bacteria. Nalidixic acid and other members of the first-generation quinolones are widely considered unattractive for extensive clinical use for a number of important reasons:(1) moderate activity against Gram-negative bacteria; (2) lack of activity against Gram-positives and pseudomonads, as well as anaerobes; (3) low blood levels for the treatment of systemic infections and skin

infections; (4) side effects produced on the central nervous system, and gastrointestinal intolerance often found when they are given at high doses to some patients; (5) bacterial resistance developed fairly rapidly resulting in frequent failure in the treatment of urinary tract infections either because of the rapid development of bacterial resistance or because of superinfection with intrinsically resistant species (Chu & Shen, 1995).

2. **Second-generation quinolones (fluoroquinolones)** include all those currently in clinical use: norfloxacin (Koga *et. al.*, 1980), pefloxacin (Goueffom *et. al.*, 1981), ciprofloxacin (Wise, Andrews & Edwards, 1983), enoxacin (Matsumoto *et. al.*, 1984), ofloxacin (Sato *et. al.*, 1982), fleroxacin (Chin, Brittain & Neu, 1986), lomefloxacin (Hokuriku Pharmaceutical Co., 1986), tosufloxacin (Chu *et. al.*, 1986; Soejima & Shimada, 1989) and sparfloxacin (Miyamoto *et. al.*, 1990).

The second-generation quinolones are extremely important in present medical treatment of bacterial infections as these drugs have a far more extensive antimicrobial spectrum, much higher potency, and more superior pharmacokinetic profile than the first-generation quinolones. Basically, the difference between these two quinolones is the addition of fluorine in the second-generation quinolone chemical structure and, as such, it is widely known as "fluoroquinolone".

Fluoroquinolones are by far the drugs of choice for treatment of infectious diseases with many advantages over other drugs. (1) There is the apparent lack of plasmid-borne resistance to the fluoroquinolones. (2) There is no evidence of hydrolysis of

fluoroquinolones by any bacterial enzymes found so far. (3) Superinfection with drug-resistant microorganisms has been rare. (4) Few side effects have been reported. (This is especially true with ciprofloxacin, the use of which has been associated with mild gastrointestinal side effects.). (5) They possess superior pharmacokinetic profiles over the first-generation quinolones (for example, higher oral bioavailability, quick distribution to the tissues and penetration into cells and body fluids, and more rapid and relatively longer serum half-life over other antibacterial agents.), and they can be administered both orally and parenterally. Because of their high potency and efficacy in oral mode of administration, peak serum concentrations are normally achieved within two hours after oral dosing. With a clinical dosage regimen of 1-2 times daily, their concentrations in serum and tissues are generally sufficiently high enough above the $MIC_{90}$ for many Gram-negative and Gram-positive aerobes, giving good clinical efficacy against simple, serious and chronic bacterial systemic infections. Thus, fluoroquinolones compare favourably with the third- and fourth-generation cephalosporins. (6) Furthermore, the majority of resistance mechanisms found to date are through chromosomal mutation that are less likely to have a major clinical impact. For these reasons, the fluoroquinolones are accepted in clinical practice as one of the most useful antibacterial drugs ever used against a good number of bacterial infections (Chu & Shen, 1995).

## CHEMICAL STRUCTURE

Quinolones generally consist of a 1-substituted-1,4-dihydro-4-oxopyridine-3-carboxylic acid moiety combined with an aromatic or heteroaromatic ring fused at 5- and 6-positions. Four-Quinolone is used as a generic name for the 4-oxo-1,4-dihydro-quinolone skeleton (Figure 1) (Chu & Shen, 1995). Thus, nalidixic acid is a 8-aza-4-quinolone (Figure 2). Related compounds are oxalic acid and pipemidic acid. Figure 3 illustrates the use of the Smith method in naming these nine ring skeletons (Smith, 1984a). Derivatives with an extra ring or rings attached to the basic skeleton at positions other than two and three are also considered as 4-quinolones (which refer to both first- and second-generation quinolones). Many clinicians and microbiologists, however, would prefer to name all of them as quinolones. Because the newer very potent quinolones possess a fluorine atom at the 6-position, they may also be referred to as fluoroquinolones : norfloxacin, pefloxacin and ciprofloxacin etc. (Figure 4) (Chu & Shen, 1995).

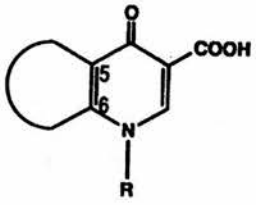


**Figure 1** General structure of quinolones (Chu & Shen, 1996).

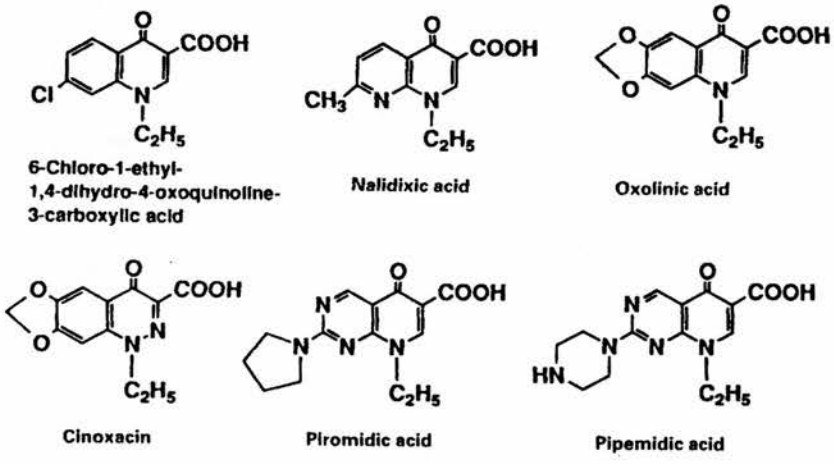


**Figure 2** First-generation quinolones (Chu & Shen, 1996).

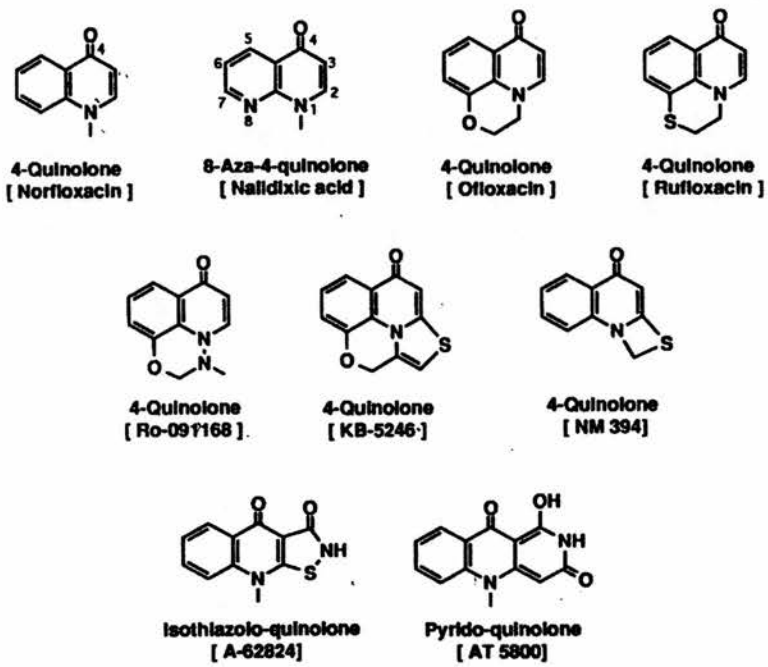


**Figure 3** Different ring skeletons of quinolones. An example of an antibacterial compound is given in brackets for each ring skeleton (Chu & Shen, 1996).

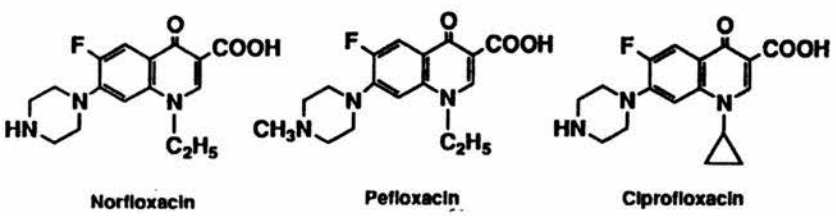


**Figure 4** Second-generation quinolones.

## DNA TOPOISOMERASE

Cell division requires topological changes are imposed upon cellular DNA prior to and during replication and segregation of chromosomes. Interconversion of different topological forms of DNA is catalysed by (DNA) topoisomerases, a family of enzymes classified according by their catalytic mechanism of action. The two predominant topoisomerases have been considered to be I and II. Type I enzymes introduce transient single stranded breaks into DNA, pass an intact single strand of DNA through the broken strand, and then re-ligate the break. Type II enzymes, in contrast, make transient double-stranded breaks into one segment of DNA and pass an intact duplex through the broken DNA, before resealing the break. Topoisomerases are ubiquitious enzymes charged with the task of resolving topological problems which arise during the various processes of DNA metabolism, including transcription, recombination, replication and chromosome partitioning during cell division (Austin & Fisher, 1990; Gasser *et. al.*, 1992; Osheroff *et. al.*, 1991; Sternglanz, 1989; Wang, 1985). As a result of performing this vital role, topoisomerase action is necessary for the viability of all organisms from unicellular bacteria to humans.

Cellular functions i.e. DNA replication, transcription from many promoters, transposition, and several types of recombination all depend on the appropriate level of supercoiling being maintained in the cell: the degree of DNA supercoiling is a major determinant of cellular functions. At the simplest level, supercoiling is a means of packaging DNA into the relatively small environment of the cell. Negative

supercoiling may be detrimental to the cell if not properly regulated by topoisomerases.

The supercoiled state of DNA is regulated by topoisomerases; therefore the enzymes are vital to function and viability of bacterial cells.

*E. coli* and probably most bacteria contain two type I and two type II topoisomerases. The two type I enzymes are topoisomerase I and topoisomerase III and they are not essential for viability (Austin & Fisher, 1990; Sternglanz, 1989; Wang, 1985). The two essential type II enzymes are DNA gyrase and topoisomerase IV (DiNardo *et. al.*, 1982; Oram & Fisher, 1992; Pruss *et. al.*, 1982; Raji *et. al.*, 1985).

DNA gyrase, an essential type II DNA topoisomerase found only in bacteria, is the primary target for quinolone action with DNA topoisomerase IV as the secondary target in many bacteria (Chu & Shen; 1995; Cozzarelli, 1980; Gellert, 1981; Reece & Maxwell, 1991). Using a gel-filtration method, the binding of [$^3$H]enoxacin to the complex formed between pBR322 DNA and serveral mutant gyrase preparations has been studied recently (Yoshida *et. al.*, 1993). This technique measured tightly-bound ligands that were not dissociated during the filtration step. The results of this study favoured the view that quinolone binds tightly only to the enzyme-DNA complex, and that both A- and B-subunits were involved with the binding (Chu & Shen. 1995). Fluoroquinolones are relatively more potent against DNA gyrase than topoisomerase IV, especially in bacterial cells (Zechiedrich *et. al.*, 1994); fluoroquinolones inhibit the activity of gyrase at concentrations lower than those inhibiting topoisomerase IV (Hooper, 1995).

In *S. aureus*, DNA topoisomerase IV has been proposed as a primary target of fluoroquinolones according to the result that mutations in QRDR of *gyrA* were not present in eight low quinolone-resistant *S. aureus* while *parC* mutations Ser-80-Phe or Ser-80-Tyr had been found in both high and low quinolone-resistant *S. aureus* (Ferrero *et. al.*, 1994). This is in agreement with Hooper (1995). Similarly, Campa *et. al.* (1997) have proposed that topoisomerase IV is the primary target of ciprofloxacin in *Streptococcus pneumoniae*, and DNA gyrase being a secondary one.

Chen *et. al.*(1996) and Heisig (1996) confirmed that GyrA is a specific primary target of quinolones and topoisomerase IV is a secondary, less sensitive target in *E. coli*. This is based on an experiment that mutant CR1 of *E. coli* with mutations at *gyrA* and *parC* (gene for topoisomerase IV) was able to grow in medium containing 0.5 mg/L ciprofloxacin while mutant GP201 of *E. coli* with only *gyrA* mutation died. The wildtype died even more quickly (Chen *et. al.*, 1996).

## DNA GYRASE

DNA gyrase almost certainly has a general role both in removing supercoils and in the introduction of negative supercoils into DNA, together with a more specialized role in removing knots and unlinking catenates generated by recombination (Adams *et. al.*, 1992).

Several studies indicate that the effects of changes in DNA supercoiling on gene expression are pervasive. DNA supercoiling can increase, decrease, or leave unaffected the expression of specific genes. Basically, in the eubacteria, negative superhelical turns (supercoiling) are introduced into DNA (relaxed and positively

supercoiled DNA) (Hooper & Wolfson, 1989) by DNA gyrase, and can be removed by one or more DNA-relaxing topoiosomerases. The enzyme carries out successive rounds of supercoiling until enough negative supercoils have been introduced to cause its dissociation. There is thus an upper limit to the degree of supercoiling which gyrase can achieve. The balance of these activities, possibly modulated also by the effects of transcription, is responsible for keeping superhelicity in an acceptable range (Menzel & Gellert, 1994). Gyrase also catalyzes the interconversion of more complex topological forms of DNA.

DNA gyrase is capable of altering the topological state of DNA in several ways: DNA replication, recombination, and transcription. Gyrase can supercoil, relax, unknot, and decatenate closed circular DNA (and reverse these latter two reactions). The enzyme's unique function is to introduce as well as remove negative superhelical turns to and from relaxed closed circular DNA. This is achieved at the expense of ATP hydrolysis (Gellert *et. al.*, 1976a). The gyrase enzyme is associated with replication forks (Drlica *et. al.*, 1980) and has access to most of the chromosome (Franco & Drlica, 1988), presumably to maintain appropriate levels of superhelical tension. These enzymes are believed to operate through a mechanism by which DNA suffers a double-strand break, and another duplex segment is passed through the break before it is resealed. Supercoiling tends to be processive, although the affinity of gyrase for DNA decreases as the negative superhelicity rises. Without ATP, gyrase relaxes negatively supercoiled DNA, but this reaction is at least 20-fold slower than supercoiling (Gellert *et. al.*, 1979; Higgnins *et. al.*, 1978). Specific amino acid changes in the gyrase subunits which are frequently associated with drug

resistance have been identified (See MECHANISMS OF RESISTANCE TO QUINOLONES, p.39).

DNA gyrase has a tetrameric structure ($A_2B_2$, MW 374,000) composed of two homodimers of subunits A (MW 97,000) encoded by the *gyrA* gene, and B (MW 90,000) encoded by the *gyrB* gene. The enzyme exists in a heart-shaped form, with the upper portion of the heart formed by the $A_2$ dimer and the B subunit attached below the A subunit (Drlica, 1984; Kirchtausen, Want & Harrison, 1985). Both genes are located far apart on the *E. coli* chromosome; *gyrA* is at 48 minutes on the standard *E. coli* map and *gyrB* is at 82 minutes. The two genes must therefore be separately controlled. In many other bacterial species the two genes are nearest neighbours (Reece & Maxwell, 1991), so close that they are presumably co-transcribed. To date, the complete nucleotide sequences of these two genes have been determined in five organisms, namely, *E. coli, Neisseria gonorrhoeae, Bacillus subtilis, Staphylococcus aureus* and *Borrelia burgdorferi* (Drlica & Kreiswirth, 1994).

## GyrA subunit

The A subunit is responsible for breaking (cutting) and rejoining (resealing) of both strands of duplex DNA. Before rejoining, a DNA segment has passed through the transient breaks. Under the control of gyrase, these reactions occur throughout relaxed or positive supercoiled DNA, which act as a substrate and become negative supercoiled DNA– a necessary form of the molecule.

As an intermediate of the reaction, the 5'-ends of the transiently broken DNA are covalently linked to a tyrosyl residue on the gyrA protein. The site of covalent attachment to DNA is Tyr-122 of the A chain (Horowitz & Wang, 1987), and the quinolone antibiotics that act on GyrA interfere with all reactions involving DNA strand passage (See MECHANISM OF QUINOLONES : DNA, RNA and protein syntheses, page 16).

In *E. coli* it has been shown that the full length protein can be divided into two separable domains: the C-terminal 300 amino acids is a DNA-binding protein, whereas the N-terminus is the cutting-rejoining domain. The N-terminal region alone is sufficient to retain DNA supercoiling activity when it is complexed with the GyrB protein (Reece & Maxwell, 1991). All sequenced *gyrA* gene homologues are 800-900 amino acids long. The highest homology is located within the N-terminal 400 amino acids, where resistant mutations usually take place.

## GyrB subunit

The B subunit is the site of ATP hydrolysis to drive the process and conformational changes in the complete enzyme to allow DNA strand passage as new molecules (Mizuuchi *et. al.*, 1978; Neu, 1994). The B subunit processes ATP; by itself, GyrB has a weak ATPase acitivity (Hsieh, 1990; Tamura & Gellert, 1990).

## DNA SUPERCOILING MODEL

Cozzarelli and his co-workers (Brown & Cozzarelli, 1979; Morrison & Cozzarelli, 1981) proposed a DNA supercoiling model consisting of the following four steps; (i) binding of gyrase to DNA substrate to stablize a positive DNA node; (ii) cleavage of DNA at 4-base pair staggered sites at the node, forming covalent linkages between a tyrosine group on the gyrase A-subunit and the 5'-end of the DNA chain; (iii) passage of the intact DNA segment at the node through the opened DNA gate, thus inverting the sign of the node; and (iv) resealing the DNA break and completing a supercoiling run.

## DNA TOPOISOMERASE IV: A POTENTIAL SECONDARY TARGET

Besides DNA gyrase, there are two other enzymes that share control of bacterial DNA topology. As DNA gyrase introduces negative supercoiling into chromosomal DNA (Drlica & Snyder, 1978; Gellert *et. al.*, 1976a, b): DNA topoisomerase I counters the action of gyrase to prevent the accumulation of excess supercoiling (DiNardo *et. al.*, 1982; Pruss *et. al.*, 1982): and DNA topoisomerase IV plays a central role resolving the catenation generated by DNA replication (Adams *et. al.*, 1992; Peng & Marians, 1993a, b). DNA topoisomerase may be membrane bound (Kato *et. al.*, 1992); thus, it may differ in their accessibility to the quinolones (Howard *et. al.*, 1993a; Urios *et. al.*, 1991).

Topoisomerase IV probably plays a very different role in chromosome topology from gyrase (Chen *et. al.*, 1996). Genetic studies indicate that topoisomerase IV is

required at the terminal stages of DNA replication, and mutants in topoisomerase IV genes are deficient in chromosomal partitioning (Adams *et. al.*, 1992; Peng & Marians, 1993b).

DNA gyrase and topoisomerase IV have amino acid sequence similarity and similar double strand passage modes of action. Both are type II enzymes and they share some similarity in their structures and functions; for example, the active form of the topoisomerase IV has a tetrameric $(ParC)_2(ParE)_2$ structure similar to the $A_2B_2$ structure of DNA gyrase (Peng & Marians, 1993b).

The bacterial topoisomerase IV gene has been found to be highly homologous to the classic type II gyrase genes: the ParE protein (601 amino acids) is homologous to the GyrB protein (52% identity), and the ParC protein (730 amino acids) is homologous to the GyrA protein (41% identity). In both cases amino acid similarities are scattered throughout the length of the proteins. The homologies, supported by biochemical evidence, leave no doubt that the ParE and ParC proteins form a complex that has topo II activity; this activity has been named topo IV (Kato *et. al.*, 1990). It is interesting to note that in the *E. coli parE* and *parC* genes, the amino acids thought to be responsible for the sensitivity of gyrase to coumarin and fluoroquinolone antibiotics are conserved in topoisomerase IV. This suggests that the ParE and ParC proteins might be sensitive to inactivation by these drugs (Huang, 1994). The DNA relaxation activity of purified *E. coli* topoisomerase IV is indeed sensitive to novobiocin and oxolinic acid inhibition, confirming the sequence predictions (Kato *et. al.*, 1992).

DNA topoisomerase IV as well as DNA gyrase are essential for cell viability and sensitive to both types of gyrase inhibitors, the coumerins and quinolones (Gellert *et. al.*, 1977; Hoshino *et. al.*, 1994; Kato & Ikeda, 1993; Peng & Marians,1993b; Sugino *et. al.*, 1977).

Ciprofloxacin was able to kill a gyrase (*gyrA*) mutant resistant to the prototype quinolone, nalidixic acid, and created complexes on DNA detected by DNA fragmentation. This lethal effect of ciprofloxacin was eliminated by additional mutations mapping in *parC*, one of the two genes encoding topoisomerase IV. Thus, the fluoroquinolone compounds have another intracellular target DNA topoisomerase IV. In the absence of the *gyrA* mutation; however, the *parC* ($Cip^R$) allele did not by itself confer resistance to ciprofloxacin, indicating that gyrase is the major quinolone target in *E. coli* (Chen *et. al.*, 1996).

## MECHANISM OF QUINOLONES : DNA, RNA and protein syntheses

All quinolones rapidly inhibit DNA synthesis (replication) and begin to inhibit RNA synthesis at a single most bactericidal concentration (See KILLING MECHANISM OF QUINOLONE, page 22; ANTIBACTERIAL ACTIVITY, page 56). Some quinolones require RNA synthesis in order to kill bacteria (See MUTUAL ANTAGONISM, page 19). Protein synthesis is required by all quinolones to kill bacteria (see below).

Trapping topoisomerases on DNA has several consequences, the most striking of which is rapid inhibition of DNA replication (Goss, Deitz & Cook, 1964). In bacteria, gyrase is clustered along the chromosome and distributed at 100 kbp intervals. When quinolones are present, complexes of quinolone-gyrase-DNA form quickly resulting in duplex DNA breakage and no functioning gyrase (Drlica *et. al.*, 1980; Engle *et. al.*, 1982). As such, DNA synthesis is inhibited in proportion to the number of complexes formed and it begins where application forks reach the complex with fork-associated gyrase. Within a few minutes after the presence of quinolone, DNA synthesis rates drop below 5% of that observed in untreated controls. Inhibition is rapid even at low drug concentrations at which inhibition is only partial and few complexes form (Snyder & Drlica, 1979).

For several quinolone compounds, inhibition of DNA synthesis occurs at the minimum inhibitory concentration for growth, which has been thought to equal minimum bactericidal concentration (Chow *et. al.*, 1988). A study demonstrated that inhibition of DNA synthesis is not by itself responsible for the bactericidal effect of oxolinic acid. Because the drug concentration sufficient to inhibit DNA synthesis by 90% in ten minutes had little effect on survival during a 2.5 hour treatment (Chen *et. al.*, 1996).

Quinolone-gyrase-DNA complex formation is not sufficient to kill cells. This result came from the study by Chen *et. al.*, (1996): no difference was observed in the size of DNA fragments generated by treatment of cells with oxolinic acid at 1 mg/L (sufficient to inhibit DNA synthesis) and 30 mg/L (sufficient to kill) when followed

by protein denaturation to release DNA ends/breaks constrained in drug-gyrase-DNA complexes, whose formation parallels inhibition of DNA synthesis with chromosomal DNA. In the other words, the same number of complexes form at both concentrations.

Neither complex fomation nor inhibition of DNA synthesis are themselves lethal, because suppression of protein or RNA synthesis during treatment with bactericidal concentrations of nalidixic acid prevents loss of colony-forming ability on agar lacking the drugs (Deitz, Cook & Gross, 1966). Collectively, these observations indicate that cell death is a consequence of cellular function(s) induced in response to the DNA-gyrase-drug interaction (Chen *et. al.*, 1996). There are some data to support the idea that intracellular release of DNA ends, not simply formation of quinolone-gyrase-DNA complexes, accounts for the lethal effect of oxolinic acid. Chen *et. al.* (1996) proved that cell death parallels the freedom of DNA strands to rotate, which is presumably is due to dissociation of the complexes, causing release of DNA breaks from constraint by gyrase. Examination of chromosomal DNA extracted from *E. coli* indicated that bacteriostatic concentrations of oxolinic acid trap gyrase and block DNA synthesis without releasing broken DNA from drug-gyrase-DNA complexes. Release occurred only at high, bactericidal oxolinic acid concentrations (Chen *et. al.*, 1996). DNA strand breakage may be the more specific cause that triggers some unknown process that kills cells [See KILLING MECHANISM OF QUINOLONE, page 22].

Release of DNA breaks and cell death were both blocked by chloramphenicol, suggesting that synthesis of additional protein activity is required to free the DNA ends (Chu & Shen, 1995). Moreover, protein synthesis-independent killing is also believed to involve in cell death caused by quinolones since rifampicin completely blocks the lethal effect of ciprofloxacin on a *gyrA* ($Nal^R$) mutant.

The more recently discovered fluoroquinolone compounds, such as ciprofloxacin, possess an additional mode of killing that does not require ongoing protein synthesis (Howard *et. al.*, 1993a, b). Ciprofloxacin at low concentration behaves much like the less potent oxolinic acid at high concentration, since chloramphenicol was very effective at blocking the bactericidal action at low concentrations of ciprofloxacin. Thus, increasing the concentration or potency of a quinolone appears to shift its mode of action from one requiring protein synthesis to one that does not. It seemed possible that the second mode of ciprofloxacin action might involve direct dissociation of quinolone-gyrase-DNA complexes.

## MUTUTAL ANTAGONISM

The use of quinolones drugs together with other drugs of different antimicrobial classes does not necessarily mean that an improvement will be found in curing bacterial infections. In fact, some of these drugs antagonise the action of quinolones and so must not be used clinically together. Considerable attention should therefore be paid to the possibility of mutual antagonism between some antibacterials and 4-quinolones. Early studies showed that the activity of nalidixic acid (Smith & Lewin, 1988) and oxolinic acid (Chen *et. al.*, 1996) were antagonized by rifampicin and by

chloramphenicol. However, the lethal effect of the potent fluoroquinolone ciprofloxacin is only partially blocked by either rifampicin or the presence of a nalidixic acid resistance ($Nal^R$) allele of *gyrA* (Lewin *et. al.*, 1991).

The 4-quinolones that are antagonised to the greatest extent by rifampicin or chloramphenicol are norfloxacin, oxolinic acid, acrosoxacin, flumequine, pipemidic acid, cinoxacin, nalidixic acid and piromidic acid. This finding came from the experiment that employed the RNA synthesis inhibitor, rifampicin which was added, to produce a final concentration of 160 mg/l, 20 min after the bacteria had been treated in nutrient broth with each 4-quinolone at its most bactericidal concentration. It completely abolished their bactericidal effects and changed the response to bacteriostasis (Smith, 1984a).

The results with ciprofloxacin and ofloxacin were in striking contrast because rifampicin addition only partially slowed their bactericidal effects. Moreover, ciprofloxacin and ofloxacin at their most bactericidal concentration more rapidly killed *E. coli* (90%, in 19 min) than any of the other 4-quinolones tested (90%, 59 mins in mean) (Smith, 1984b). Rifampicin also has no significant effect on the activity of the newer fluoquinolone temafloxacin and sparfloxacin, (Smith & Lewin, 1988; Neu, 1991).

Therefore, 4-quinolones can be divided into two groups according their susceptibility to antagonism by RNA synthesis inhibition.

1. Group A. Rifampicin-sensitive 4-quinolones: norfloxacin, oxolinic acid, acrosoxacin, flumequine, pipemidic acid, cinoxacin, nalidixic acid and piromidic acid.

2. Group B. Rifampicin-insensitive 4-quinolones: ciprofloxacin, ofloxacin, temafloxacin and sparfloxacin

Group A can cause a secondary inhibition of RNA synthesis which has mechanism A (common to all 4-quinolones) which can be abolished by rifampicin addition. Group B has not only A but mechanism B (unique to ofloxacin and ciprofloxacin) which is completely resistant to rifampicin antagonism. In other words, group B was not able to, more or less, cause the secondary inhibition or its bactericidal activity is not as susceptible to antagonism by RNA synthesis inhibition.

Group B drugs appear to have an additional mode of action. They can kill non-growing cells, cells in stationary phase, and cells treated with inhibitors of protein and RNA syntheses (Zeiler, 1985; Lewin *et. al.*, 1990; Eng *et. al.*, 1991). Non-induced lethality requires higher concentrations of drug than does the induced form (Lewin *et. al.*, 1991), and it is not exerted by all compounds against all bacteria; for example, ciprofloxacin, which exhibits non-induced killing of *E. coli*, does not kill staphylococci in the presence of chloramphenicol, while ofloxacin does (Lewin & Smith, 1988). The mechanism of non-induced killing of bacteria is not understood at present (Drlica & Kreiswirth, 1994).

Synergy has been noted infrequently; for example for a combination of anti-*Pseudomonas* penicillins, anti-*Pseudomonas* cephalosporins, and imipenem with the fluoroquinolones against *P. aeruginosa* (Neu, 1991). Synergy is rarely seen with aminogylcosides. Combination of fluoroquinolones with clindamycin, erythromycin, metronidazole, and vancomycin does not affect the activity against aerobic or anaerobic Gram-positive or Gram-negative bacteria. The activity of the fluoroquinolones against Enterobacteriaceae is decreased in the presence of chloramphenicol (Smith & Lewin, 1988).

## KILLING MECHANISM OF QUINOLONE

Mechanisms of 4-quinolones have been divided into three (Howard, Pinney & Smith, 1994):

Mechanism A: The only mechanism, possessed by the original 4-quinolone nalidixic acid, requires cellular replication and RNA and protein syntheses, what may result from the induction of SOS processing. Since mechanism A requires RNA synthesis, it is inhibited by rifampicin, and since it requires cell division , it is inactive in washed bacteria suspended in phosphate-buffered saline (PBS), in which cell division is inhibited.

Mechanism B: It is possessed by ciprofloxacin and ofloxacin, and requires neither RNA nor protein synthesis and is active against non-dividing bacteria. Mechanism B is not inhibited by rifampicin, and is effective on cells suspended in PBS (Zeiler & Grohe, 1984).

Mechanism C: It is possesed by ciprofloxacin, ofloxacin and norfloxacin, and kills non-dividing bacteria, but requires protein and RNA syntheses. Therefore it will occur in cells suspended in PBS, but is inhibited by rifampicin (Ratcliffe & Smith, 1985).

The presently available information offers only partial insight of the underlying causes of cell death caused by the topoisomerase-targeting drugs. High-affinity gyrase-quinolone interaction sites on the chromosome are poorly understood (Drlica & Kreiswirth, 1994). A major determinant of their cytotoxicity is the conversion of a latent single- or double-stranded break in a drug-topoisomerase-DNA ternary complex into an irreversible double stranded DNA break. It is beleived to lead to DNA relaxation, activation of the SOS response and degradation of DNA. (Herrero & Moreno, 1986).

Quinolones (at bacteriostatic concentrations and above) trap DNA gyrase on broken chromosomal DNA and act as a catalytic intermediate. This results in stabilised DNA-drug-gyrase complexes containing DNA breaks and thus prevents enzyme turnover, DNA synthesis and cell growth (Gellert *et. al.*, 1977; Sugino *et. al.*, 1977). Consequently, supercoiling, relaxation, catenation, and decatenation are all inhibited, but ATP hydrolysis is not appreciably affected. Saturating concentrations of quinolone result in cleavage of the chromosome into about 50-100 pieces (Bejar & Bouche, 1984; Snyder & Drlica, 1979), although only a small fraction of the DNA is cleaved at the 50-100 high-frequency interaction sites (toposites) detected by pulsed-field gel electrophoresis (Condemine & Smith, 1990).

The stabilized complex, termed a 'cleavable complex', is believed to trigger a cellular process leading to cell death (Drlica, 1984; Kreuzer & Cozzarelli, 1990). This complex has both DNA strands cleaved with a 4-bp stagger and the 5' ends protruding (Gellert *et. al.*, 1977; Sugino *et. al.*, 1977; Morrison & Cozzarelli, 1979). The 5'-phosphoryl end at the break is covalently bound to the A subunit (Morrison & Cozzarelli, 1979). As such, the specific target of the antibacterial quinolone is the A-subunit of DNA gyrase (Chu & Shen, 1995).

When purified DNA gyrase and DNA are treated with oxolinic acid and then with detergents, such as sodium dodecyl sulfate, site-specific breakage of duplex DNA is observed with one gyrase A subunit attached to the 5' end of each of the two severed DNA strands. Incubation of gyrase and DNA in the presence of a quinolone drug and termination of the reaction with SDS leads to double-stranded cleavage of the DNA and covalent attachment of the A subunits to the 5'-phosphate groups at the break sites (Drlica, 1984; Hooper *et. al.*, 1986; Gellert *et. al.*, 1977; Sugino *et. al.*, 1977; Morrison & Cozzarelli, 1979). Specifically, a phosphate ester is formed between Tyr122 of GyrA and the 5'-end of the DNA (Horowitz & Wang, 1987). Thus, it is thought that quinolones lead to the interruption of the supercoiling cycle at the DNA breakage-rejoining step.

Although the A sub-unit does appear to bind DNA in the absence of the B sub-unit (Kirchhausen, Wang & Harrison, 1985), and the B sub-unit is found to have a weak ATPase activity, the ATPase activity of the gyrase B protein is only fully stimulated in the presence of the A protein and DNA (Maxwell & Gellert, 1984). Whereas the A

protein is the target of quinolone drugs, the B protein seems to be the target of both 4-quinolone and coumarin drugs such as novobiocin. Further evidence has been shown by Yamagishi *et. al.* (1986) that two quinolone-resistant mutations in the *E. coli gyrB* gene have been identified at the DNA sequence level suggesting that both subunits of gyrase may be a target for quinolone attack. A more specific target of action of 4-quinolones has been shown to be the A subunits. However, mutations in gyrB have secondary effects on quinolone interaction mediated via protein-protein contacts. Other resistant mutations have also been mapped, for example *norB, norC, nfxB* and *cfxB* which decrease the expression of the porin outer membrane protein OmpF (Hirai *et. al.,* 1986; Hooper *et. al.,* 1986, 1989).

The mechanism of inhibition by quinolone on DNA gyrase at the molecular level has not been elucidated completely. In 1989 Shen and his coworkers proposed the cooperative quinolone-DNA binding model as illustrated in Figure 5 (Shen *et. al.*, 1989). This model proposed that the drug binding site is induced during the gate-opening step of the supercoiling process. The separated short DNA strands between

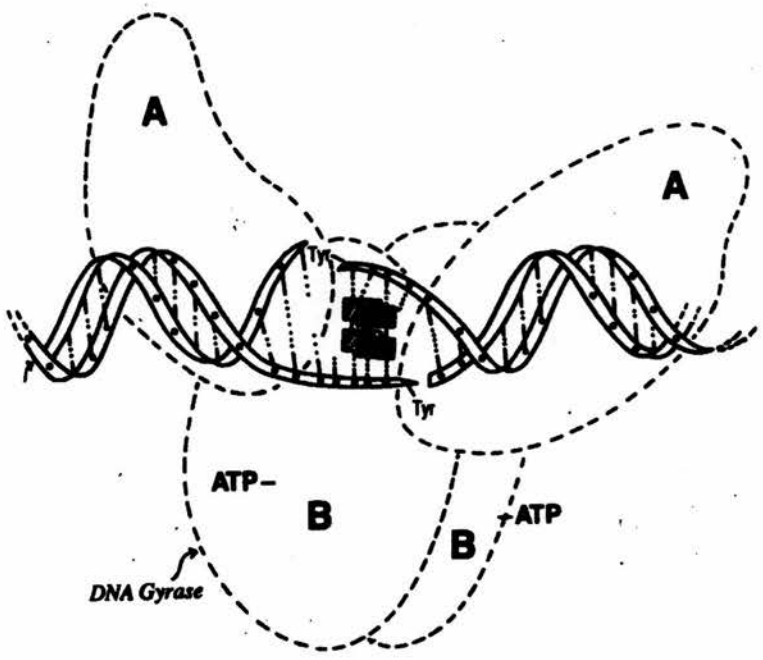


**Figure 5** **Quinolone-DNA co-operative binding model for the inhibition of DNA gyrase. Quinolone molecules (filled and slashed rectangles at the centre of the diagram) bind to a gyrase-induced DNA site during the intermediate gate-opening step of the DNA supercoiling process. Binding of the drug is through hydrogen-bonding (indicated by dotted lines) to the unpaired bases. The stacking of the rectangles mimics the mode of drug self-association shown at the right. The tyrosine-122 residues of the gyraseA-subunits are covalently linked transiently to the 5'-end of the DNA chain (Horowitz & Wang, 1987) during the supercoiling catalysis. Subsequent opening of the DNA chains, between the four-base-pair staggered cuts, results in a locally denatured DNA bubble which is proposed to be the site for drug binding. The binding of the drug molecules thus stabilizes the enzyme-DNA intermediate. When relaxed DNA substrate (represented by the short DNA segment in the diagram) is used, ATP is required for the induction of the drug binding site. Dashed curves denote the shape of the DNA gyrase, a tertrameric toposiomerase composed of two A-subunits and two B-subunits, as revealed by the electron microscopic image of the *Mycobacterium luteus* enzyme (Shen *et. al.*, 1989).**

the 4-base-pair staggered cuts may form a denatured DNA bubble that is a preferred site for the drug to bind in a multi-dimensional fashion. Quinolones, with their simple molecular structure and few functional binding groups, acquire high-binding affinity via self-association of the drug molecules. Two types of interactions have been proposed on the basis of nalidixic acid crystal structures: the $\pi$-$\pi$ stacking between the quinolone rings and the tail-to-tail hydrophobic interactions between the N-1 substitution groups (Figure 5, right panel). Such interactions not only enhance the binding strength but also provide flexible adjustment in fitting the receptor conformation in the binding pocket.

Quinolones are essentially DNA-targeted drugs. They possess two unique characteristics at the molecular level: they do not bind to the isolated target enzyme, and they bind preferentially to single-stranded rather than double-stranded DNA. Participation of the DNA-bound enzyme in creating the binding site and the capability of the drug to saturate the binding pocket in a co-operative manner account for the drug's specficity and superior potency.

It is likely that gyrase A subunit as a primary target to ciprofloxacin is not sensitive to inhibition of protein synthesis but topoisomerase IV ParC subunit is (Chen *et. al.*,1996). This was based on the evidence that, in the presence of 0.5 mg/L ciprofloxacin, mutant GP201 (*gyrA* ($Nal^R$) mutation) became fully resistant on treatment with chloramphenicol, and the wildtype was partially protected by chloramphenicol. Since there was a distinct lag before killing, by ciprofloxacin, commenced with the *gyrA* ($Nal^R$) mutant strain GP201, the lethal effects associated

with topoisomerase IV were manifested slowly (Chen *et. al.*, 1996). Introduction of the *lexA-3* allele, which prevents induction of the SOS response, eliminated the lag: thus, one or more components of the SOS response retard the lethal effects of ciprofloxacin-topoisomerase IV interactions (Chen *et. al.*, 1996).

Chu and Shen (1995) found that ten fluoroquinolones had MIC values that are normally one or two orders of magnitude lower than the inhibitory activities against the purified enzyme. In their experiments, the antibacterial activity and the DNA gyrase inhibition activity of ten fluoroquinolones had good correlation, with the antibacterial concentrations on the average about 20-fold lower than the anti-gyrase concentrations. As such, they concluded that at least three factors may contribute to this phenomenon: (1) the drug is concentrated in the cell by an active transport process, (2) a small signal produced at sub-$IC_{50}$ concentration is sufficient and can be amplified by some unknown cellular process leading to the cell killing, and (3) there are secondary targets in the cell responsible for the lethality. For each quinolone, one or more of the above factors may contribute to its bactericidal action against different bacterial species including resistance mutants (Chu & Shen, 1995).

Factor (1) is unlikely as it would require a specific quinolone concentration mechanism, which would be paradoxical for a completely synthetic compound. Factor (2) is more likely–the $I_{50}$ is an arbitary figure and it is much more likely that the concentration to inhibit the cell is going to be far lower than the $I_{50}$, when half the action of the enzyme is annihilated.

## SELECTIVITY FOR BACTERIA, NOT FOR HUMANS

Quinolones have toxic effects on bacterial DNA gyrase and topoisomerase IV, but not on human DNA topoisomerases. This selectively is because there are fundamental differences as to how their DNA is organized. Bacterial chromosome is not bounded by a nuclear envelope as is the case in humans. In the human cell, the eucaryotic nucleus has 46 separate chromosomes. Each chromosome is composed of chromatin, half of which is small basic proteins, termed histones, which are firmly associated with an equal weight of DNA. In contrast, bacteria have no histones and only one chromosome, which is very different in structure from that in humans. Human cells possess a topoisomerase II which, like bacterial DNA gyrase, can transiently cut and seal ds DNA by introducing two nicks staggered apart by four base pairs with the 5' end protruding (Miller, Liu & Englund, 1981; Liu *et. al.*, 1983). This enzyme, unlike bacterial DNA gyrase, is composed of two subunits (rather than four) each of MW 172k. Even more significantly, the mammalian enzyme does not possess any negative supercoiling activity. Therefore, it is not surprising that the human enzyme is insusceptible to inhibition by the 4-quinolone antibacterials (Miller *et. al.*, 1981): quinolones drugs are generally toxic to only bacteria, not to humans.

## CLINICAL USE

Fluoroquinolones are found to be highly effective for the treatment of both complicated and uncomplicated urinary tract infections, as well as for acute and chronic prostatitis, bacterial gastroenteritis, enteric fevers and selected respiratory infection (Turnidge, 1995). They are considered as the drug of choice for the

management of gastrointestinal tract infections. Moreover, they are very effective for the treatment of acute pyelonephritis due to the fact that high concentrations can be achieved in both the kidney and urine. They have also been demonstrated to be highly efficacious for the treatment of sexually-transmitted diseases, Gram-negative bacillary osteomyelitis, bone and joint and skin and skin-structure infections, and can be used in the single-dose treatment of uncomplicated gonococcal urethritis and cervicitis. Multiple doses are needed for the effective treatment of genital infections with *Chlamydia trachomatis* or *Ureaplasma urealyticum* and single-dose treatment for these infections is not recommended. The clinical and bacteriological cure rates for fluoroquinolone therapy of respiratory tract infections are as high as 90% (Chu & Shen, 1995).

Pneumonia caused by *Streptococcus pneumoniae*, and infections caused by enterococci are not effectively treated with fluoroquinolones with the exception of sparfloxacin and tosufloxacin, owing to their less potent activity *in vitro* against these bacteria. Furthermore, fluoroquinolone have limited activities against a number of clinically-important Gram-positive bacteria such as *Streptoccocus pyogenes* and, particularly, methicillin-resistant *S. aureus* and *P. aeruginosa* (Blumberg *et. al.*, 1991; Parry *et. al.*, 1989). For pelvic inflammatory disease, fluoroquinolones (with the possible exception of tosufloxacin and sparfloxacin) should not be used as a single agent for the treament of this kind of infection (Chu & Shen, 1995).

Among fluoroquinolones, ciprofloxacin was shown by Smith (1986) that it stands out clearly as the most active antibiotic not only because of its average minimum

inhibitory concentration (MIC) against 21 mutants which was as little as 0.22 mg/L, but also because the mutant most resistant to it was inhibited by 0.5 mg/L which is well within its peak serum level of about 3 mg/L following a 0.5 g oral dose (Hooper & Wolfson, 1985).

Nalidixic acid and similar first-generation quinolones are clinically used principally for the treatment of two types of infection: in developed countries, treatment of lower urinary tract infection; in developing countries, treatment of bacterial diarrhoeas (Turnidge, 1995).

## ANTIMICROBIAL SPECTRUM

Fluoroquinolones are highly effective in curing many types of bacterial infections. Therefore, many bacterial diseases that were difficult to treat with the first-generation quinolones, and even other classes of antibacterial drugs, have been easily controlled with fluoroquinolones.

Among the commercially-available quinolones whose *in vitro* activity are various, ciprofloxacin is the most active agent (Barry, 1990). Tosufloxacin has activity which is quite similar to that of ciprofloxacin, and a compound which does not yet have a name, OPC-17116, has activity very similar to that of ciprofloxacin against the Enterobacteriaceae (Espinoza *et. al.*, 1988; Neu *et. al.*, 1992). Among the quinolones used clinically, only tosufloxacin and sparfloxacin possess reasonable activity against anaerobes. In general, norfloxacin, ofloxacin, lomefloxacin, and fleroxacin have bery similar antimicrobial profiles, whereas sparfloxacin, tosufloxacin, and

ciprofloxacin are more active agents (Chin *et. al.*, 1991). Susceptibility for enterobacterial organisms usually differs little from one agent to another by, at most , one- or two-fold dilutions.

All of the commercially available fluoroquinolones have excellent activity in vitro encompassing not only Gram-negative aerobes but also many Gram-positive aerobes as well as some anaerobes. Inherently, fluoroquinolone-sensitive bacteria comprise the majority of the Enterobacteriaceae causing not only urinary or gastrointestinal diseases but also respiratory, skin structure, and bone and joint infections. These organisms are *Escherichia coli, Klebsiella pneumoniae, Enterobacter* spp., *Serratia marcescens, Salmonella* spp., *Shigella* spp., and *Campylobacter* spp. The antibiotics also have superior activity against fastidious Gram-negative species such as *Haemophilus influenzae* and other *Haemophilus* species, *Neisseria gonorrhoeae, Neisseria meningitidis,* and *Moraxella (Branhamella) catarrhalis* (Barry, 1990; Eliopoulos & Eliopoulos, 1989; Neu, 1989).

In 1988 it would have been possible to state that virtually 99% of members of the aforementioned organisms would have been susceptible to <2 mg/L. However, by 1991 resistance was seen among *Klebsiella* species, *Enterobacter* species, *Serratia marcescens,* and occasional *Providencia* species. Nonetheless, currently 90% of community isolates of Enterobacteriaceae would be inhibited by concentrations of > 2 mg/L (Neu, 1994).

Fluoroquinolones have good activity against Gram-positive bacteria: *Staphylococcus aureus* and many of the coagulase-negative staphylococci (Barry, 1990; Eliopoulos & Eliopoulos, 1989; Neu, 1992). These include *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, and the urinary pathogen *Staphylococcus saprophyticus*. In the 1980s the MICs of fluoroquinolones against staphylococci, including methicillin-resistant staphylococci, ranged between 0.25 and 2 mg/L. Unfortunately, since 1989 methicillin-resistant *S. aureus* resistant to fluoroquinolones have been encountered throughout the world (Blumberg *et. al.*, 1991; Kaatz *et. al.*, 1991; Isaacs *et. al.*, 1988; Maple *et. al.*, 1989; Schaeffer, 1989; Shalit *et. al.*, 1989). These strains have minimum inhibitory concentrations (MICs) in the range of 4-32 mg/L. Compounds such as tosufloxacin and sparfloxacin inhibit staphylococci at lower concentrations (i.e., 0.12 mg/L) and will inhibit a number of the methicillin-resistant *S. aureus* (MRSA) resistant to ciprofloxacin at concentrations of 1 mg/L (Chin *et. al.*, 1991). Although many streptococci have high MICs, most staphylococci, including methicillin-resistant isolates, are susceptible. In addition, fluoroquinolones are able to kill beta-lactamase-containing bacteria, and those resistant to aminoglycosides. Fluoroquinolones are also active against *Mycobacterium tuberculosis* with sparfloxacin the most active agent (Neu *et. al.*, 1992). Fluoroquinolones, in general, inhibit *P. aeruginosa* at concentrations that are achievable in urine. Ciprofloxacin has been the most active agent, and prior to 1988 it inhibited 90% of *P. aeruginosa* at concentrations of <1 mg/L. In recent years many *P. aeruginosa* isolates have ciprofloxacin MICs in the range of 2-8 mg/L (Neu, 1994). Of the common genital pathogens, fluoroquinolones are very active against *Neisseria gonorrhoeae* and *Haemophilus ducreyi*, but are only moderately active

against mycoplasmas, ureaplasmas and chlamydiae. They are found to have activity against *Rickettsia conovic*, *M. tuberculosis* and *M. leprae*, as well as *M. avium* (Neu, 1994).

Ciprofloxacin is a new fluoroquinolones that is highly active against a broad array of microbial pathogens. MICs of ciprofloxacin are generally below 0.5 mg/L for *Hemophilus*, *Neisseria*, and *Enterobacteriaceae* and are 1.0 mg/L or less for many non-fermentative Gram-negative bacteria. Most staphylococci, including strains resistant to methicillin, are inhibitied by 1.0 mg/L or less of ciprofloxacin, whereas streptococci are somewhat less susceptible. Obligate anaerobes are generally not susceptible to ciprofloxacin at concentrations below 1.0 mg/L. Factors diminishing the *in vitro* activity of ciprofloxacin include acidic pH, high levels of magnesium ions, and an inoculum size of $10^7$ CFU/ml or greater. Table 1 shows the *in vitro* activities of ciprofloxacin against Gram-positive bacteria.

**Table 1** **The *in vitro* activities of ciprofloxacin against Gram-positive bacteria.**

| **Bacteria** (Numbers of strains) | **MIC (mg/L)** |
|---|---|
| *Staph. epidermidis* (20) | 0.03-0.5 |
| *Staph. aureus* (20) | 0.12-1.0 |
| *Strep. pyogenes* (20) | 0.25-2.0 |
| *Strep. pneumoniae* (20) | 0.5-16 |
| Group B streptococci (10) | 0.5-2.0 |
| Viridans streptococci (30) | 1.0-16.0 |
| Enterococci (20) | 0.25-2.0 |
| *H. influenzae* (20) | ≤0.007-0.03 |
| *N. meningitidis* (20) | ≤0.007-0.03 |
| *N. gonorrhoeae* (20) | ≤0.007-0.03 |
| *E. coli* (10) | ≤0.007-0.05 |
| *Shigella* spp. (10) | ≤0.007-0.06 |
| *Salmonella* spp (10) | ≤0.007-0.015 |
| *Klebsiella* spp. (10) | 0.03-0.25 |
| *Proteus mirabilis* (10) | ≤0.007-0.12 |
| *Enterobacter* spp. (10) | ≤0.007-0.06 |
| *Citrobacter* spp. (10) | ≤0.007-0.25 |
| *Serratia* spp. (10) | ≤0.007-0.12 |
| *Providencia stuartii* (20) | ≤0.007-0.5 |
| *Morganella morganii* (30) | ≤0.007-0.015 |
| *P. aeruginosa* (20) | 0.06-8.0 |
| Other non-fermenters (27) | ≤0.007-1.0 |
| Other non-fermenters (3) | 2.0-4.0 |

## CLINICAL DEVELOPMENT OF RESISTANCE

Resistance is only rarely encountered among common pathogens. In most studies, 97 to 100% of all pathogens are fully susceptible to fluoroquinolones (Goldstein & Acar, 1995). Nevertheless, a clinical resistant clone may be selected during therapy and cause clinical failure. It may or may not disseminate, depending on epidemiological conditions (Goldstein & Acar, 1995). However, with the extensive general use worldwide of the fluoroquinolones, resistance is now being seen in Enterobacteriaceae, including *E. coli* and *Klebsiella* species. Resistance to fluoroquinolones in the clinical setting has been seen principally with methicillin-resistant *S. aureus* and *P. aeruginosa* (Blumberg *et. al.*, 1991; Parry *et. al.*, 1989; Wolfson & Hooper, 1989). Fluoroquinolone resistance has followed treatment of cutaneous infections, osteomyelitis, and respiratory infections, particularly in cystic fibrosis patients (Bosso, 1989; Peterson *et. al.*, 1989; Scully *et. al.*, 1987). In urinary tract infections resistance has been seen in *Serratia marcescens, Ent.* spp., and *E. coli* as well (Fujimaki *et. al.*, 1989). Ciprofloxacin-resistant MRSA have been noted in Europe, the United States, and Japan. Studies have indicated that within a short period up to 80% of MRSA in an institution may be resistant to all of the fluoroquinolones (Maple *et. al.*, 1989; Soussy *et. al.*, 1991). In 1991, the New York City Department of Health Phage Typing Laboratory received more than 4000 MRSA isolates from more than 30 different New York City health care facilities. Almost 90% were ciprofloxacin resistant (Drlica & Kreiswirth, 1994). Some investigators have reported 95 to 100% fluoroquinolone resistance among MRSA (Goldstein & Acar, 1995). In addition, methicillin-susceptible *S. aureus* resistance

has been increasing. Soussy *et. al.* (1991) noted that 92% of MRSA had by 1990 developed resistance to pefloxacin, and 22% of methicillin-susceptible *S. aureus*. Most reports of the incidence of fluoroquinolone-resistant *S. aureus* do not distinguish between methicillin-resistant *S. aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA), or indicate the origin of the strain. More than 96% of MRSA isolated from community-acquired infections are susceptible to fluoroquinolones (Acar & Francoual, 1990; Acar *et. al.*, 1993; Hoban & Jones, 1993; Jones, 1992; Jones *et.al.*, 1992; Thornsberry, Brown & Bouchillon, 1992). 38% of *P. aeruginosa* and 54% of *S. marcescens* were found to be resistant to pefloxacin. High-level resistance to fluoroquinolones (i.e., MIC of ciprofloxacin [Cip] greater than 1 mg/L) is rarely found among clinical isolates of *E. coli* (Barry *et. al.*, 1990). In Japan there has been a 20-30% increase in fluoroquinolone resistance in Gram-negative species such as *P. aeruginosa* (Tabuko, 1992). However, a longitudinal study over 5 years in the United Kingdom reported that 91% of *P. aeruginosa* remained sensitive to Cip (Tillotson, Culshaw and O'Keefe, 1992) and in Germany, ofloxacin resistance amongst Gram-negative species as a whole totalled less than 4% after 7 years's use (Focht, Kraus & Noser, 1992). Further, despite the free availability of both ethical and "bootleg" preparations of norfloxacin and ciprofloxacin in South India, where most of the population harbour trimethoprim-ampicillin- and chloramphenicol-resistant commensals, there was no cross-resistance to fluoroquinolones amongst nalidixic acid-resistant aerobic faecal flora (Amyes *et. al.*, 1991).

The most common mechanisms of resistance to fluoroquinolones appears to be alterations in the DNA gyrase A subunit and, in the case of Gram-negatives,

alteration of outer membrance proteins and lipopolysaccharide (Cohen *et. al.*, 1989; Daikos *et. al.*, 1988; Hirai *et. al.*, 1987). It is also the alterations of *parC* of topoisomerase IV that is responsible for low and high resistance to fluoroquinolones in some bacteria (Heisig, 1996).

Studies with *E. coli* have implicated several genes in resistance to the quinolones. Those in the gyrase genes are the best studied. The *gyrA* (*nalA*) gene encodes the A subunit of gyrase. Mutations in *gyrA* are commonly associated with quinolone resistance in clinical isolates (Nakamura *et. al.*, 1989; Reece & Maxwell, 1991) and are responsible for high levels of drug resistance (Hane & Wood, 1969; Gellert *et. al.*, 1977; Sugino *et. al.*, 1977). In *E. coli* these mutations cluster around Ser-83 in the N-terminal portion of *gyrA* gene (Yoshida *et. al.*, 1990a). Such mutants can be found clinically. In general, changes in the *gyrA* gene produce an eight- to 16-fold increase in the concentration of a fluoroquinolone required to inhibit or kill bacteria (Yoshida *et. al.*, 1990a). In Gram-negative organisms the *gyrA* gene which confers susceptibility to fluoroquinolones appears to be dominant over *gyrA* resistance genes so that resistance does not occur through genetic transfer of plasmids carrying *gyrA* gene (Weidemann, 1990). It does if the recipient becomes a *gyrA* homozygote by Hfr-like transfer.

Examination of amino acid changes conferring resistance indicated that in *S. aureus* resistance arises from mutation of *gyrA*, as it does in *E. coli* (Sreedharn *et. al.*, 1990), with amino acid changes occurring in equivalent positions in the proteins. The

staphylococci probably also exert resistance through restricted uptake of the quinolones, as has been found with *E. coli* (*nalB* and *nalD*).

Resistance to quinolones can also be caused by changes in permeability. Aoyoma *et. al.* (1988) described decreased expression of OmpF due to changes in the *nfxB* and *cfxB* genes which prevented accumulation of fluoroquinolones in cells, as normally occurs in an energy-dependent fashion. Permeation and DNA gyrase mutations have been described in *P. aeruginosa* and *Citrobacter freundii* (Aoyama *et. al.*, 1988; Celesk & Robillard, 1989). Two additional loci appear to affect the cell permeability the quinolones. One is *nalB* (*E. coli* map position, 57 minuties), which provides low-level resistance (Hane & Wood, 1969), and the other is *nalD* (*E. coli* map position, 89.8 minutes), which confers a higher level of resistance (Hrebenda *et. al.*, 1985). The latter locus is sometimes confused with another called *nalD*, that was subsequently mapped to *gyrB* (Yamagishi *et. al.*, 1981, 1986).

Of particular concern is the use of fluoroquinolones in veterinary medicine; enrofloxacin is used in most European countries in virtually all animals. In Japan, up to 6 different fluoroquinolones are very widely used in veterinary practice. The US Food and Drug Adminstration (FDA) is currently preparing some very restrictive measures for governing the use of fluoroquinolones in animals (FDA, 1994).

## MECHANISMS OF RESISTANCE TO QUINOLONES

It was an unusual finding and rather surprising that the frequency of clinical resistance to the quinolones is much less than that seen with the other major groups

of antibiotics and chemotherapeutic agents. This mainly results from the fact that no plasmids have been found to be involved in the 4-quinolone resistance and this contrasts markedly with resistance to almost all other antibacterial drugs (Turnidge, 1995). Furthermore, fluoroquinolones inhibit transfer of large plasmids (Weiser & Weidemann, 1987). However, a Japaneses group proposed a plasmid mediated quinolone resistance in MRSA. Another report confirmed that resistance does not occur through genetic transfer as to the finding that the *gyrA* gene which confers susceptibility to fluoroquinolones appears to be dominant over *gyrA* resistance genes in Gram-negative organisms (Weidemann, 1990). Thus, only chromosomal mutation is responsible for clinical resistance and even that is unusual in the clinical situation; indeed it is rarely found, because the spontaneous mutation rate is low at $10^{-9}$ to $10^{-12}$.

Unfortunately, with the extensive general use worldwide of the fluoroquinolones, resistance has been found clinically, particularly with methicillin-resistant *S. aureus* and *P. aeruginosa* (Blumberg *et. al.*, 1991; Parry, Pazer & Yokna, 1989) and in Enterobacteriaceae for which these agents had been considered to be a major breakthrough. An example of careless use of quinolones is the extensive use of enoxacin, ofloxacin, and lomefloxacin for pneumonia or bacterial bronchitis when *S. pneumoniae* is the aetiological agent. This organism responds poorly and continuous challenge will certainly increase the development of quinolone-resistant strains.

Strains resistant to nalidixic acid are significantly more resistant to the new fluoroquinolones than are nalidixic acid-susceptible strains. However, most of these strains remain within the range of susceptibility to fluoroquinolones (Bryskier, 1993;

Wolfson & Hooper, 1989). In Gram-negative bacilli, fluoroquinolones resistance is most often a two-stage process, with resistance to previous generations of quinolones such as nalidixic acid emerging first, conferring higher minimum inhibitory concentrations for the fluoroquinolones, but usually staying in the susceptible range. This is followed by further mutations to generate full resistance to the fluoroquinolones. It is possible that widespread resistance to nalidixic acid was already established in developing countries, which facilitated the subsequent emergence of resistance to fluoroquinolones (Hooper & Wolfson, 1990). There is evidence showing such a trend in the increasing proportion of nalidixic acid-resistant *E. coli* that are becoming fluoroquinolone resistant in the Western Pacific region (Turnidge, 1995).

The mechanisms of quinolone resistance mediated via chromosomal mutations include (1) alteration of outer membrane proteins and/or changes in lipopolysaccharide in case of Gram-negatives (Cohen *et. al.*, 1989; Daikos *et. al.*, 1988; Hirai *et. al.*, 1987), (2) increased efflux of quinolones, (3) changes in permeability and (4) a decreased affinity of DNA gyrase (Daniel & Shen, 1995). The latter is indeed the major cause of resistance giving altered amino acids mostly in a specific region of *gyrA* or *parC* genes.

GyrA protein is the main target of inactivation by quinolone antibiotics. A change in a single amino acid in the protein due to mutation in the *gyrA* gene has been shown to be associated with resistance (Cullen *et. al.*, 1989; Dimri & Das, 1990; Hopewell *et. al.*, 1990; Korten, Wai & Murray, 1994; Tankovic *et. al.*, 1996). These mutations

are clustered within a narrow region between *E. coli* nucleotides 382 (Ala-67) and 501 (Gln-106) (Swanberg & Wang, 1987; Yoshida *et. al.*, 1990a, b and c; Hallett & Maxwell, 1991). Therefore, this region, which is highly conserved in various bacteria, is designated as the quinolone resistance-determining region (QRDR) of the *gyrA* gene (Yoshida *et. al.,* 1990a). This region is in the vicinity of the active site of the gyrase A subunit, Tyr-122.

Genetic characterisation by determining the DNA sequences of the respective *gyrA* genes coding for the A subunit of DNA gyrase of several strains with low-level resistance to fluoroquinolones (MIC of Cip <1 mg/L) revealed 10 different point mutations within QRDR at residues Ala-67, Gly-81, Ser-83, Ala-84, Asp-87 and Gln-106, that can confer fluoroquinolone drug resistance in *E. coli* (Cullen *et. al.*, 1989; Hallett & Maxwell, 1991; Oram & Fisher, 1991; Yoshida *et. al.*, 1988; Yoshida *et. al.*, 1990a). The most critical amino acid changes associated with high levels of fluoroquinolone resistance are located in residues Ser-83 to Asp-87 of the *E. coli* protein (Hallett & Maxwell, 1991; Oram & Fisher, 1991; Yoshida *et. al.*, 1990a, b and c).

## MUTATION AT 83 AND 87

Many mutants isolated from patients have had changes in the *E. coli*'s Ser-83 and Asp-87 residues in the *gyrA* gene associated with a significant increase in the resistance towards all quinolones (Oram & Fisher, 1991; Ruiz *et. al.*, 1995; Yoshida *et. al.*, 1990a). In general, the changes produce an eight- to 16-fold increase in the

concentration of a fluoroquinolone required to inhibit or kill bacteria (Yoshida *et. al.*, 1990a).

In *E. coli* it has been demonstrated that a single mutation of Ser-83-Ala in the *gyrA* gene is sufficient to render the DNA gyrase activity resistant to ciprofloxacin and nalidixic acid *in vitro* when the mutant GyrA protein is reconstituted with the wild-type GyrB protein (Hallett & Maxwell, 1991). The high-level quinolone resistance of *gyrA*'s Ser-83 *E. coli* mutants and the weaker binding of quinolone to the complex formed between DNA and the mutant gyrase compared with that between DNA and the wild-type gyrase, have prompted the proposal that Ser-83 is a direct quinolone-binding site mediated through a hydrogen bond (Maxwell, 1992). The commonest mutation at this position is Ser-83-Leu which is responsible for low-level resistance to fluoroquinolones (i.e., MIC of ciprofloxacin $\leq$ 1 mg/L) in both laboratory mutants and clinical isolates of *E. coli* (Cullen, 1989; Fisher *et. al.*, 1989; Oram & Fisher, 1991; Piddock, 1995; Yoshida *et. al.* 1988). Table 2 summarizes reported single mutations at several position in *gyrA*'s and *parC*'s QRDR of *E. coli*, associated with Cip resistance.

The importance of Ser-83 was more in its position rather than amino acid Ser itself. The position 83 was proposed by Yonezawa *et. al.* (1995a), that it is the most important for the 83rd amino acid residue to be hydrophobic in expressing the phenotype of quinolone resistance. The researchers also suggested that the hydroxyl group of Ser does not play a major role in the quinolone-gyrase interaction as to their

result that Ser-83-Gly, -Asn, -Asp, and -Lys mutations in *gyrA* expressed sensitivity to quinolones and the Ser-83-Tyr mutation conferred resistance to quinolone.

**Table 2 Single mutations in *gyrA's* and *parC's* QRDR of some *E. coli* with MIC of Cip (mg/L), and *E. coli*'s *gyrA* homologous position.**

| Bacteria | Position in *E. coli's gyrA*[a] | Mutations[b] | MIC of Cip | Reference |
|---|---|---|---|---|
| *E. coli* | – | *GyrA*: none | 0.0125 | Yoshida *et. al.* (1988) |
| *E. coli* | 67 | *GyrA*: Ala-67-Ser | 0.05 | Yoshida *et. al.* (1988) |
| *E. coli* | 67 | *GyrA*: Ala-67-Ser | 0.2 | Yoshida *et. al.* (1990a) |
| *E. coli* | 81 | *GyrA*: Gly-81-Cys | 0.1 | Yoshida *et. al.* (1990a) |
| *E. coli* | 81 | *GyrA*: Gly-81-Asp | 16 | Cambau *et. al.* (1992) |
| *E. coli* | 83 | *GyrA*: Ser-83-Trp | 0.25 | Cullen *et. al.* (1989) |
| *E. coli* | 83 | *GyrA*: Ser-83-Trp | 0.25 | Oram & Fisher (1991) |
| *E. coli* | 83 | *GyrA*: Ser-83-Trp | 0.5 | Yoshida *et. al.* (1990a) |
| *E. coli* | 83 | *GyrA*: Ser-83-Trp | 16 | Yoshida *et. al.* (1990a) |
| *E. coli* | 83 | *GyrA*: Ser-83-Leu | 16 | Yoshida *et. al.* (1988) |
| *E. coli* | 83 | *GyrA*: Ser-83-Leu | 0.5 | Yoshida *et. al.* (1990a) |
| *E. coli* | 83 | *GyrA*: Ser-83-Leu | 0.25 | Oram & Fisher (1991) |
| *E. coli* | 83 | *GyrA*: Ser-83-Ala | 4.0 | Hallett & Maxwell (1991) |
| *E. coli* | 84 | *GyrA*: Ala-84-Pro | 0.1 | Yoshida *et. al.* (1990a) |
| *E. coli* | 84 | *GyrA*: Ala-84-Pro | 4.0 | Yoshida *et. al.* (1990a) |
| *E. coli* | 87 | *GyrA*: Asp-87-Asn | 0.25 | Yoshida *et. al.* (1990a) |
| *E. coli* | 87 | *GyrA*: Asp-87-Asn | 4.0 | Yoshida *et. al.* (1990a) |
| *E. coli* | 87 | *GyrA*: Asp-87-Val | 0.05 | Oram & Fisher (1991) |
| *E. coli* | 87 | *GyrA*: Asp-87-Tyr | 8.0 | Heisig *et. al.* (1993) |
| *E. coli* | 106 | *GyrA*: Gln-106-His | 0.05 | Yoshida *et. al.*(1988) |
| *E. coli* | 106 | *GyrA*: Gln-106-Arg | 0.05 | Hallett & Maxwell (1991) |
| *E. coli* | – | *ParC*: none | 0.39 | Kumagai *et. al.* (1996) |
| *E. coli* | 81 | *ParC*: Gly-78-Asp | 25 | Kumagai *et. al.* (1996) |
| *E. coli* | 83 | *ParC*: Ser-80-Ile | 12.5 | Kumagai *et. al.* (1996) |
| *E. coli* | 83 | *ParC*: Ser-80-Arg | 3.13-12.5 | Kumagai *et. al.* (1996) |
| *E. coli* | 87 | *ParC*: Glu-84-Lys | 12.5 -25 | Kumagai *et. al.* (1996) |

[a] Homologous amino acid position in *gyrA* of *E. coli*, where a mutation has occurred.
[b] "*GyrA*: Ser-83-Leu" indicates amino acid Ser at position 83 of *gyrA* is changed to Leu.
(Note that none of all isolates had both their *gyrA* and *parC* QRDR sequenced)

Asp-87 is the second-most common of the resistance mutations of *E. coli gyrA* gene. The Asp-87-Gly mutation is a novel mutation leading to the loss of a negatively charged residue in the QRDR of the gyrase A protein. In this respect, it resembles the Asp-87-Val mutation found in a clinical isolate of *E. coli* (Oram & Fisher, 1991) and on Asp-87-Thr mutation identified in a one-step mutant of *E. coli* K-12 JM83 selected *in vitro*. The latter resulted in an increase in the MIC of Cip from 0.015 to 0.25 mg/L. Glu-87-Gly was associated with high-level resistance to Cip (MICs, 8 and 16 mg/L) in *Coxiella burnetii* (Musso, *et. al.*, 1996).

Yonezawa *et. al.*, (1995b) reported that Asp-87-Ala, -Val, -Phe, -Ser, -Asn and -Lys mutations in *gyrA* gene of *E. coli* conferred resistance to quinolones and observed that Asp-87-val and Asp-87-Asn were discovered in clinical isolates and laboratory mutants. The researchers suggested that the carboxyl group of Asp-87 may interact with the quinolone drug. Table 3 summarises some more examples of bacteria and their single mutations at 83 and 87 associated with Cip resistance.

Recently, mutations at both Ser-83 and Asp-87 have been shown to occur together giving rise to a higher MIC of 64 mg/L of ciprofloxacin than in strains with only 1 mutation. Mutations at Ser-83 and Asp-87 is responsible for high-level resistance to fluoroquinolones (Heisig *et. al.*, 1993; Huang, 1992).

**Table 3 Single mutations in *gyrA's* QRDR of some bacteria with MIC of Cip,and their *E. coli*'s *gyrA* homologous position.**

| Bacteria[a] | Position in *E. coli*'s *gyrA*[b] | Mutations[c] | MIC of Cip | Reference |
|---|---|---|---|---|
| *E. faecalis* | – | *GyrA*: none | 8 | Tankovic *et. al.* (1996) |
| *E. faecalis* | 83 | *GyrA*: Ser-83-Arg | 64 | Tankovic *et. al.* (1996) |
| *E. faecalis* | 83 | *GyrA*: Ser-83-Ile | 32-64 | Tankovic *et. al.* (1996) |
| *E. faecalis* | 87 | *GyrA*: Glu-87-Gly | 32-64 | Tankovic *et. al.* (1996) |
| *E. faecalis* | – | *GyrA*: none | 8-16 | Korten, Wai & Murray (1994) |
| *E. faecalis* | 83 | *GyrA*: Ser-83-Arg/Ile | 32-128 | Korten, Wai & Murray (1994) |
| *E. faecalis* | 87 | *GyrA*: Glu-87-Lys | 32-128 | Korten, Wai & Murray (1994) |
| *E. faecium* | – | *GyrA*: none | 32 | Korten, Wai & Murray (1994) |
| *E. faecium* | 83 | *GyrA*: Ser-83-Arg/Ile | 32-128 | Korten, Wai & Murray (1994) |
| *E. faecium* | 87 | *GyrA*: Glu-87-Lys | 32-128 | Korten, Wai & Murray (1994) |
| *S. typhimurium* | – | *GyrA*: none | 0.003-0.06 | Ouabdesselam *et. al.*(1996) |
| *S. typhimurium* | 83 | *GyrA*: Ser-80-Tyr | 0.25-1 | Ouabdesselam *et. al.*(1996) |
| *S. enteritidis* | – | *GyrA*: none | 0.03-0.06 | Ouabdesselam *et. al.*(1996) |
| *S. enteritidis* | 83 | *GyrA*: Ser-80-Phe | 0.25-1 | Ouabdesselam *et. al.*(1996) |
| *S. hadar* | – | *GyrA*: none | 0.03-0.06 | Ouabdesselam *et. al.*(1996) |
| *S. hadar* | 83 | *GyrA*: Ser-80-Phe | 0.25-1 | Ouabdesselam *et. al.*(1996) |
| *A. baumannii* | – | *GyrA*: none | 1 | Vila *et. al.* (1995) |
| *A. baumannii* | 83 | *GyrA*: Ser-83-Leu | ≥4 | Vila *et. al.* (1995) |

[a] Full name of bacteria: *Enterococcus faecalis*, *Enterococcus faecium*, *Salmonella typhimurium*, *Salmonella enteritidis*, *Salmonella hadar*, *Acinetobacter baumannii*,

[b] Homologous amino acid position in *gyrA* of *E. coli*, where mutations occurred.

[c] "*GyrA*: Ser-83-Leu" indicates amino acid Ser at position 83 of *gyrA* is changed to Leu.

(Note that none of all isolates had both their *gyrA* and *parC* QRDR sequenced.)

The typical current resistant situation due to chromosomal mutation in *gyrA* and *parC* of *E. coli* is likely to be that found by Heisig (1996) who stated that "In fifteen strains of *E. coli* with MICs of ciprofloxacin (Cip) between 0.015 and 256 mg/L examined for the presence of mutations in the QRDR of the *gyrA* and *parC* genes, no mutation was found in a susceptible isolate (MIC of Cip, 0.015 mg/L). Four moderately resistant strains (MIC of Cip 0.06 to 4 mg/L) carried one *gyrA* mutation affecting Ser-83, but in only one of these strains was an additional *parC* mutation (Gly-78-Asp) detected. All ten highly resistant strains (MIC of Cip, >4 mg/L) examined carried two *gyrA* mutations affecting residues Ser-83 and Asp-87, and at least one *parC* mutation. These *parC* mutations included alterations of Ser-80 to Arg or Ile and Glu-84 to Gly or Lys."

Table 4 summarises double and more mutations at *gyrA* and *parC* 83 and 87 detected in *E. coli*. In the table there were two *E. coli* with highest ever-reported Cip MIC of 128 mg/L. One of them was a clinical isolate found in Spain by Vila *et. al.* (1996) and had mutations at *gyrA* and *parC* 83 and 87. The other *E. coli* was a laboratory-produced strain by Heisig *et. al.* (1993) and had mutations at *gyrA* 83 and 87 and no report on *parC* sequence.

**Table 4** **Double or more mutations at 83 and 87 in *gyrA's* and *parC's* QRDR of clinical *E. coli* with MIC of Cip, and their *E. coli*'s *gyrA* homologous position.**

| Bacteria | Position in *E. coli*'s *gyrA*[a] | Mutations[b] | MIC of Cip | Reference |
|---|---|---|---|---|
| *E. coli* | – | *GyrA*: none | 0.007-0.25 | Vila *et. al.*, (1996) |
| *E. coli* | 83 | *GyrA*: Ser-83-Leu | 0.25 | Vila *et. al.*, (1996) |
| *E. coli* | 83<br>83 | *GyrA*: Ser-83-Leu,<br>*ParC*: Ser-80-Arg | 1-4 | Vila *et. al.*, (1996) |
| *E. coli* | 83<br>83<br>87 | *GyrA*: Ser-83-Leu,<br>*ParC*: Ser-80-Ile<br>*ParC*: Glu-84-Val | 2 | Vila *et. al.*, (1996) |
| *E. coli* | 83<br>87 | *GyrA*: Ser-83-Leu,<br>*ParC*: Glu-84-Lys | 4 | Vila *et. al.*, (1996) |
| *E. coli* | 83<br>87<br>87 | *GyrA*: Ser-83-Leu,<br>*GyrA*: Asp-87-Tyr,<br>*ParC*: Glu-84-Lys | 8-32 | Vila *et. al.*, (1996) |
| *E. coli* | 83<br>87<br>87 | *GyrA*: Ser-83-Leu,<br>*GyrA*: Asp-87-Asn,<br>*ParC*: Glu-84-Lys | 8 | Vila *et. al.*, (1996) |
| *E. coli* | 83<br>87<br>83 | *GyrA*: Ser-83-Leu,<br>*GyrA*: Asp-87-Asn,<br>*ParC*: Ser-80-Ile | 8-64 | Vila *et. al.*, (1996) |
| *E. coli* | 83<br>87<br>83 | *GyrA*: Ser-83-Leu,<br>*GyrA*: Asp-87-Asn,<br>*ParC*: Ser-80-Arg | 16 | Vila *et. al.*, (1996) |
| *E. coli* | 83<br>87<br>83<br>87 | *GyrA*: Ser-83-Leu,<br>*GyrA*: Asp-87-Asn,<br>*ParC*: Ser-80-Ile,<br>*ParC*: Glu-84-Val | 64 | Vila *et. al.*, (1996) |
| *E. coli* | 83<br>87<br>83<br>87 | *GyrA*: Ser-83-Leu,<br>*GyrA*: Asp-87-Tyr,<br>*ParC*: Ser-80-Ile,<br>*ParC*: Glu-84-Lys | 128 | Vila *et. al.*, (1996) |

[a] Homologous amino acid position in *gyrA* of *E. coli*, where a mutation has occurred.
[b] "*GyrA*: Ser-83-Leu" indicates amino acid Ser at position 83 of *gyrA* is changed to Leu.

Mutations at Ser-91-Phe in *GyrA* gene and Ser-87-Ile in *parC* gene conferred clinically significant resistance to fluoroquinolones (MIC of ofloxacin, 8.0 mg/L; MIC of ciprofloxacin, 1.0 mg/L) in *N. gonorrhoeae*. In wild-type bacteria, amino acid numbering of GyrA protein can vary from one bacterium to another and so as to the type of corresponding amino acid within QRDR; for example, Ala-84 and Asp-87 in *E. coli* are Ser-85 and Glu-88, respectively, in *S. aureus*.

Huang (1992) reported that there was a correlation between fluoroquinolone susceptibility and different amino acids at *GyrA* positions 83, 84, and 87 in wild-type bacteria of different species. Organisms whose *gyrA* gene has Ser at position 83, Ala at position 84, and Asp at position 87 are most sensitive to fluoroquinolone (as is the case with *E. coli*). This level can be modulated by different amino acids at these three positions. The drug sensitivity is increased one order of magnitude by having residues of Ser at 84 and Glu at 87, as in *S. aureus* (Huang, 1994). The like amino acid Thr at position 83 instead of Ser may be sufficient to raise the drug sensitivity by an order of magnitude, as in the cases of *Klebsiella pneumoniae* and *Campylobacter jejuni*. The intrinsic resistances of *C. jejuni* and *K. pneumoniae* are high at $MIC_{90}$ of 0.25-0.5 mg/L. The presence of amino acids other than Ser or Thr at the 83 position appears to render bacteria resistance to fluoroquinolone drugs at even higher levels (Huang, 1992). The drug resistance to one or two orders of magnitude above the intrinsic level is again conferred if the wild-type amino acid at the position equivalent to *E. coli GyrA* 83 in *S. aureus* and *C. jejuni* is changed to a different amino acid, namely, Ser-83-Ala and Thr-83-Ile, respectively (Sreedharan *et.*

*al.*, 1990). In *B. burgdorferi* Gln(Q) is present at Ser-83, and its $MIC_{90}$ for Cip is about 1 mg/L (Huang, 1994). Some other bacteria and mutations at position 83 and 87 assosiated with Cip resistance are summarised in Table 5 and with norfloxacin or ofloxacin resistance in Table 6.

**Table 5** **Mutations in *gyrA*'s and *parC*'s QRDR of some bacteria with MIC of Cip (mg/L), and *E. coli*'s *gyrA* homologous position.**

| Bacteria | Position in *E. coli's gyrA*[a] | Mutations[b] | MIC of Cip | Reference |
|---|---|---|---|---|
| *E. cloacae* | – | *GyrA*: none,<br>*ParC*: none | ≤0.025 | Deguchi *et. al.* (1997b) |
| *E. cloacae* | 83 | *GyrA:* Ser-83,<br>*ParC*: none | 0.39-3.13 | Deguchi *et. al.* (1997b) |
| *E. cloacae* | 83 | *GyrA:* Ser-83,<br>*ParC*: Glu-84 | 3.13 | Deguchi *et. al.* (1997b) |
| *E. cloacae* | 83 & 87<br>– | *GyrA*: Ser-83 & Asp-87<br>*ParC*: none | 25 | Deguchi *et. al.* (1997b) |
| *E. cloacae* | 83 & 87<br>83 & 87 | *GyrA:* Ser-83 & Asp-87<br>*ParC*: Ser-80 or Glu-84 | 25-100 | Deguchi *et. al.* (1997b) |
| *S. aureus* | –<br>83 | *GyrA*: none,<br>*ParC*: Ser-80-Tyr | 2-32 | Ferrero, Cameron & Crouzet (1995) |
| *S. aureus* | –<br>87 | *GyrA*: none,<br>*ParC*: Glu-84-Lys | 2-32 | Ferrero, Cameron & Crouzet (1995) |
| *S. aureus* | 87<br>83 | *GyrA*: Glu-88-Lys,<br>*ParC*: Ser-80-Tyr | 32 | Ferrero, Cameron & Crouzet (1995) |
| *S. aureus* | –<br>83 | *GyrA*: none,<br>*ParC*: Ser-80-Tyr | 32 | Ferrero, Cameron & Crouzet (1995) |
| *S. aureus* | 83<br>83 | *GyrA*: Ser-84-Leu,<br>*ParC*: Ser-80-Tyr | 128 | Ferrero, Cameron & Crouzet (1995) |
| *S. aureus* | 83<br>87 | *GyrA*: Ser-84-Leu,<br>*ParC*: Glu-84-Leu | 128 | Ferrero, Cameron & Crouzet (1995) |
| *N. gonorrhoeae* | –<br>– | *GyrA*: none,<br>*ParC*: none | ≤0.015 | Deguchi *et. al.* (1997a) |
| *N. gonorrhoeae* | 87<br>– | *GyrA*: Asp-95-Asn,<br>*ParC*: none | 0.126 | Deguchi *et. al.* (1997a) |
| *N. gonorrhoeae* | 83<br>– | *GyrA*: Ser-91-Tyr,<br>*ParC*: none | 0.126 | Deguchi *et. al.* (1997a) |
| *N. gonorrhoeae* | 83<br>- | *GyrA*: Ser-91-Phe,<br>*ParC*: none | 0.06-0.5 | Deguchi *et. al.* (1997a) |
| *N. gonorrhoeae* | 83<br>82 | *GyrA*: Ser-91-Phe,<br>*ParC*: Asp-86-Asn | 0.25-0.5 | Deguchi *et. al.* (1997a) |
| *N. gonorrhoeae* | 83<br>83 | *GyrA*: Ser-91-Phe,<br>*ParC*: Ser-87-Ile | 0.5 | Deguchi *et. al.* (1997a) |
| *N. gonorrhoeae* | 83<br>87<br>84 | *GyrA*: Ser-91-Phe,<br>*GyrA*: Asp-95-Asn,<br>*ParC*: Ser-88-Pro | 2.0 | Deguchi *et. al.* (1997a) |
| *N. gonorrhoeae* | 83<br>87<br>87 | *GyrA*: Ser-91-Phe,<br>*GyrA*: Asp-95-Gly,<br>*ParC*: Glu-91-Gly | 8.0 | Deguchi *et. al.* (1997a) |

[a] Homologous amino acid position in *gyrA* of *E. coli*, where a mutation is occurred.
[b] "*GyrA*: Ser-83-Leu" indicates amino acid Ser at position 83 of *gyrA* is changed to Leu.

**Table 6** **Mutations in gyrA's QRDR of some bacteria with MIC of norfloxacin (Nor) and ofloxacin (Ofx), mg/L, and E. *coli*'s *gyrA* homologous position.**

| Bacteria[a] | Position in *E. coli*'s *gyrA*[b] | Mutations[c] | MIC[d] | Reference |
|---|---|---|---|---|
| *N. gonorrhoeae* | – | *GyrA*: none | Nor:: 0.004-0.06 | Tanaka *et. al.*(1996) |
| *N. gonorrhoeae* | 83 | *GyrA*: Ser-91-Phe | Nor: 1.0-8.0 | Tanaka *et. al.*(1996) |
| *M. smegmatis* | 83 | *GyrA*: Ala-91-Val | Ofx: 8.0 | Revel *et. al.* (1994) |
| *M. smegmatis* | 87 | *GyrA*: Asp-95-Gly | Ofx: 8.0 | Revel *et. al.* (1994) |
| *M. smegmatis* | 83 | *GyrA*: Ala-91-Val | Ofx: 32-64 | Revel *et. al.* (1994) |
| *M. smegmatis* | 87 | *GyrA*: Asp-95-Gly | Ofx: 32-64 | Revel *et. al.* (1994) |
| *M. smegmatis* | 83<br>87 | *GyrA*: Ala-91-Val,<br>*GyrA*: Asp-95-Gly | Ofx: 32-64 | Revel *et. al.* (1994) |
| MRSA | – | *GyrA*: none | Ofx: <12.5 | Tanaka *et. al.* (1995) |
| MRSA | 83 | *GyrA*: Ser-84-Leu | Ofx: 12.5- ≥50 | Tanaka *et. al.* (1995) |
| MRSA | 87 | *GyrA*: Glu-88-Lys | Ofx: 12.5- ≥50 | Tanaka *et. al.* (1995) |
| MSSA | – | *GyrA*: none | Ofx: <12.5 | Tanaka *et. al.* (1995) |
| MSSA | 87 | *GyrA*: Glu-88-Lys | Ofx: <12.5 | Tanaka *et. al.* (1995) |

[a] Bacteria: *Mycobacterium smegmatis*, methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-susceptible *Staphylococcus aureus* (MSSA).

[b] Homologous amino acid position in *gyrA* of *E. coli*, where a mutation has occurred.

[c] "*GyrA*: Ser-83-Leu" indicates amino acid *Ser* at position 83 of *gyrA* is changed to Leu.

[d] MICs of norfloxacin (Nor) or ofloxacin (Ofx), mg/L.

## MUTATION AT OTHER POSITIONS

Two different one-step mutations in the *gyrB* gene of gyrase were detected in *E. coli* KL16:Asp-426-Asn resulting in a decrease in the susceptibilities to acidic quinolones but an increase in those to amphoteric quinolones, while Lys-447-Glu resulted in a decrease of the susceptibilities to all quinolones (Yamagishi *et. al.*, 1986; Yoshida *et. al.*, 1991). Some mutations map in *gyrB* which are not of much account (Yamagishi *et. al.*, 1986).

## HOMOLOGOUS AMINO ACIDS AND THEIR POSITIONS IN *gyrA* AND *parC*

DNA gyrase and topoisomerase IV are homologous. However, there is variation in size of these two enzymes in different bacteria. Therefore, an amino acid can have a different position number and that causes confusion. For example, Glu-88 in GyrA of *S. aureus* can be assigned as position 87 according to its homologous position in *E. coli* and the latter position is mentioned by several authors. It is also more communicable to refer position in ParC protein to its homologous in *E. coli*'s GyrA protein when their functions are considered; one number is refered to. Moreover, in case a complete sequence of *gyrA* or *parC* is not obtained as in this study, it is impossible to number positions according to individual bacterium. It is accordingly advisable to observe the homologous comparison of these amino acid residues between different bacteria and the two genes.

Table 7 shows some homologous positions of bacteria that are different in number and type of amino acids. (In the results and discussions of this study, *gyrA* and *parC* positions are numbered according to its *E. col* homologous positions.)

**Table 7 Homologous comparision of some amino acids and their positions in *gyrA* and *parC* of some bacteria.**

| Bacteria | Gene | Homologous Amino acids and its position | | | |
|---|---|---|---|---|---|
| *Escherichia coli* | *GyrA* | Ala-67 | Ser-83 | Asp-87 | Ser-97 |
| *Staphylococcus aureus* | *GyrA* | Ala-68 | Ser-84 | Glu-88 | Asn-98 |
| *Nisseria gonorrhoeae* | *GyrA* | Ala-75 | Ser-91 | Asp-95 | Ala-105 |
| *Escherichia coli* | *ParC* | Ala-64 | Ser-80 | Glu-84 | Ser-94 |
| *Staphylococcus aureus* | *ParC* | Ala-64 | Ser-80 | Glu-84 | Lys-94 |
| *Nisseria gonorrhoeae* | *ParC* | Ala-71 | Ser-87 | Glu-91 | Thr-101 |

## TOPOISOMERASE IV

Topoisomerase IV is a secondary, less sensitive target for quinolone action in *E. coli* and that the development of high-level fluoroquinolone resistance in *E. coli* requires at least one *parC* mutation in addition to the *gyrA* mutation (see MUTATION AT 83 AND 87, page 42) (Heisig, 1996).

## EPIDEMIOLOGY OF QUINOLONE RESISTANCE

Overall, fluoroquinolone resistance levels in both non-pathogens and pathogens are highest in developing countries and lowest in developed countries, with transitional countries undergoing rapid economic improvement showing intermediate levels of resistance (Turnidge, 1995). In developed countries, levels of resistance to fluoroquinolones exceeded 10% for only *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Acinetobacter baumannii* and *Providencia* species. Turnidge (1995) found that resistance levels of 25% or more in *Escherichia coli* were noted in three countries in 1993. In contrast, resistant strains of *S. typhi* and *S. paratyphi* A were

rare or nonexistent in any country, and only low levels of resistance were detected in *Shigella* species.

According to Turnidge (1995), fluroquinolone resistance rates are higest in the developing countries (Thailand, China, Fiji, the Philippines, Vietnam), moderate in transitional countries (Hong Kong, Singapore, Malasia, Korea, Breunei), and lowest in developed countries (Japan, New Zealand and Australia). Levels of resistance in many common pathogens has occurred: such as *E. coli*, hospital-acquired Gram-negative bacilli, such as *Enterobacter* and *Acinetobacter* spp. already at worrying levels in China, Korea, the Philippines and Singapore; invasive salmonellae (*S. typhi* and *S. paratyphi* A) at present very low, with the exception of *S. paratyphi* A in the Philippines in 1993. Low levels of resistance to fluoroquinolones are now being detected in *Shigella* spp. in a number of countries. Resistance in *Staphylococccus aureus* is now becoming an obvious problem in many countries. As suggested by previous experience in the United States, this is strongly associated with strains that are multiresistant to many antimicrobial agents, including methicillin/oxacillin. Similar resistance levels are being seen in coagulase-negative staphylococci. At present, only one country (Australia) has noted resistance in *Neisseria gonorrhoeae*, and this is at a low level.

Ciprofloxacin-resistant MRSA have been noted in Europe, the United States, and Japan. Studies have indicated that within a short period up to 80% of MRSA in an institution may be resistant to all of the fluoroquinolones (Maple *et. al.*, 1989; Soussy *et. al.*, 1991). In addition, methicillin susceptible *S. aureus* resistance has been

increasing. Soussey *et. al.* (1991) noted that 92% of MRSA had by 1990 developed resistance to perfloxacin, and 22% of methicillin-susceptible *S. aureus*. They also found that 38% of *P. aeruginosa* and 54% of *S. marcescens* were resistant to pefloxacin.

## ANTIBACTERIAL ACTIVITY

Interestingly, one feature of all 4-quinolones is that they always give a biphasic bacterial survival curve when a given bacterium had been treated with 4-quinolones over a range of concentrations during a single time interval. The drugs usually display a single most bactericidal concentration, the exact value of which varies depending on types of bacteria tested, the 4-quinolones used and other conditional factors. On either side of this concentration, greater or smaller, there is less bacterial death. Thus even as the concentration increases above the most bactericidal concentration, the ability of the drug to kill the bacterium deteriorates. It was revealed that this second phase of the 4-quinolone's activity, where the drugs become gradually less bactericidal, exists because they begin to inhibit RNA synthesis.

It was generally concluded that; among four 4-quinolones, the most efficient 4-quinolone drug to kill bacteria is ciprofloxacin; the next most efficient are ofloxacin and norfloxacin and the least is nalidixic acid. Among these drugs, the most bactericidal concentration of only nalidixic acid far exceeds its serum concentration - that of ciprofloxacin, ofloxacin and norfloxacin are readily attainable in the serum during therapy (Hooper & Wolfson, 1985). As a result, no resistant mutant was encountered that was resistant to peak serum concentrations of ciprofloxacin,

norfloxacin or ofloxacin, or resistant to attainable urine concentrations of these bactericidal agents (Smith, 1986).

# 2. MATERIALS AND METHODS

2.1 Reagents

2.2 Growth Media and Buffers

2.3 Bacteria

2.4 Storage of Bacteria

2.5 Growth of Bacteria

2.6 Determination of the Minimum Inhibitory Concentration (MIC)

2.7 Identification of Clinical Bacterial Isolates

2.8 Genetic characterisation of Mutation in the QRDR of *gyrA* and *parC* Genes of Clinical Cip-Resistant Bacteria

2.8.1 Preparation of Chromosomal DNA by Enzyme Lysis plus Solvent Extraction

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2.8.3 Verification of DNA Product by Agarose Gel Electrophoresis

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2.8.5 DNA-Strand Separation

1. Preparation of Dynabeads
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4. Separating the DNA Strands

2.8.6 DNA Sequencing

2.8.7 DNA Sequencing-Gel Electrophoresis and Autoradiography

# 2. MATERIALS AND METHODS

## 2.1 REAGENTS

Most chemicals and reagents were supplied by Sigma Chemicals (Poole, Dorset). All antimicrobial agents were freshly prepared before use with the appropriate solvent listed below along with their abbreviations and the manufacturers. Others were shown and described in the appropriate context.

**Table 8 Details of antimicrobial agents used in this study**

| ANTIMICROBIAL AGENT | ABBRE–VIATION | SOLVENT | MANUFACTURER |
|---|---|---|---|
| Chloramphenicol | Cm | Ethanol | Sigma |
| Ciprofloxacin | Cip | Water | Byer |
| Enoxacin | Eno | 0.1 M HCl | Sigma |
| Nalidixic acid | Nal | Water | Sigma |
| Norfloxacin | Nfx | 0.1 M HCl | Merck, Sharp and Dohme |
| Ofloxacin | Ofx | 0.5 M NaOH | Roussel Laboratories Ltd |
| Oxolinic acid | Oxl | 0.5 M NaOH | Sigma |
| Rifampicin | Rif | Water | Ciba Laboratories |

## 2.2 GROWTH MEDIA AND BUFFERS

1) Columbia Base Agar (Oxoid, Basingstoke, Hants.) was used routinely to grow all bacteria overnight except where stated.

2) Iso-Sensitest (IST) Broth/Agar (Oxoid, Basingstoke, Hants.) with antimicrobial agents if required, was used routinely to grow bacteria for viable counts in determination of MIC of all bacteria except *Neisseria* spp. and *Moraxella* spp.

that required Columbia Base Agar (Oxoid, Basingstoke, Hants.) with 5.% (v/v) defribrinateted horse blood.

3) CLED media (Amersham plc., UK).

4) 0.85 % NaCl in distilled water was used for bacterial dilution.

5) 10X TAE buffer pH7.6 (40 mM Tris-acetate, 1 mM EDTA).

6) Binding & Washing Buffer (10 mM Tris-HCl, pH7.5, 1 mM EDTA and 2.0 M NaCl (final concentration: 1.0 M)).

7) TE Buffer (10 mM Tris-HCl, pH7.5 and 1 mM EDTA).

## 2.3 BACTERIA

1) *Escherichia coli* NCTC10418.
2) *Escherichia coli* KL16 obtained from Dr R.J.Pinney, School of Pharmacy, University of London.
3) *Escherichia coli* clinical isolate obtained from Edinburgh Royal Infirmary Hospital.
4) *Staphylococcus aureus* NCTC6571.
5) *Pseudomonas aeruginosa* NCTC10662.
6) Clinical Quinolone-resistant isolates from Malaysia (10 isolates) and Thailand (13 isolates).

## 2.4 STORAGE OF BACTERIA

For long-term storage, all bacteria were grown to log phase of growth and then were put in tight-sealed polystyrene tubes (maximum volume of 1.5 ml) containing 1 ml nutrient medium and 20 % (v/v) glycerol, and stored in the deep freezer at a constant temperature of -70$^0$C. To revive the culture, cells were transferred by inoculation loop and placed on agar to check for purity. If there was difficulty in reviving the culture, it was grown overnight in broth before any further test.

## 2.5 GROWTH OF BACTERIA

A single colony from recently streaked agar plates was routinely inoculated into sterile liquid medium and grown overnight under sterile conditions before passing to the next stage. In the preparation of logarithmic phase cultures, the bacteria were regularly grown overnight at 37$^0$C and 130 rpm in a rotary shaker to ensure good, even growth. Subsequently, 0.1 ml was subcultured into a new universal tube containing 9.9 ml Iso-Sensitest broth and incubated overnight at 37$^0$C and 130 rpm in orbital shaker.

When a viable count was performed a liquid culture, 100 µl was serially diluted in 0.85% NaCl solution. 100 µl of suitable dilution were spread on Columbia Base Agar plate and incubated at 37$^0$C. When the bacteria under test were *Neisseria* spp. or *Moraxella* spp., a $CO_2$ incubator was required in order to provide a precise temperature of 37$^0$C.

## 2.6 DETERMINATION OF THE MINIMUM INHIBITORY CONCENTRATION (MIC)

Minimum Inhibitory Concentrations (MICs) were performed on Columbia Base Agar following the British Society for Antimicrobial Chemotherapy (BSAC) guidelines for susceptibility testing where applicable (Phillips, 1991). MICs were determined by agar double-dilution plate method. The procedure is as shown below.

1. Inoculum preparation: One fresh single colony of the bacterium to be tested was put into a Mc Cartney bottle containing 9.9 ml Iso-Sensitest broth and then incubated by shaking overnight at $37^0$C. 0.1 ml of the culture was then inoculated to another 9.9 ml Iso-Sensitest broth and incubated for 16 hours at $37^0$C before dilution in 0.85% sterile saline to give an inoculum of $1x10^4$ colony forming units (cfu/spot). The concentration of bacteria was estimated by measuring its optical density at 550 nm carried out in a spectrophotometer. The calculation was based on each bacterial standard curve of optical density and viable cell count. The range of optical density chosen was from the straight part of the curve only, in order to give accurate numbers of viable cells.

2. MIC plate preparation: A series of 12.5 ml Iso-Sensitest agar plates with doubling concentrations of an antibiotic were prepared.

    2.1 A correct amount of 10 and/or 1000 mg/l antibiotic solution was added to empty plates with appropriate label and identification marks, to produce the final concentration needed for each set of experiments.

2.2 An amount of 12.5 ml of 50$^0$C Iso-Sensitest agar was added to each plate.

2.3 To make a good homogeneous distribution of antibiotic added, the plates were rotated and any bubbles were removed with a quick exposure of flame from a Bunsen burner. After solidification the plates were dried in 55$^0$C incubator for about 30 minutes with the lids opened.

3. Inoculation: The labelled agar plates were aseptically inoculated with a 2 µl spot of each fresh-prepared inoculum, delivered from a multipoint inoculator (Denley, Billinghurst, Surrey).

4. Incubation and result observation: Inoculated agar plates were incubated at 37$^0$C, and the result was observed the next day when the control showed a correct result: for Cip, MIC of *Escherichia coli* NCTC10418 was 0.016 mg/L; *Pseudomonas aeruginosa* NCTC10662, 0.12 mg/L; *Staphylococcus. aureus* NCTC6571, 0.12 mg/L.

Note: In the case of inoculum preparation of *Neisseria* spp. and *Moraxella* spp. that require addition of blood and precise temperature for growth, cells were grown on Columbia Base Chocolate agar at precisly 37$^0$C in $CO_2$ incubator, and one loopful of fresh culture was diluted and mixed well in 0.85% NaCl to give the same standard cell number of inoculum. MIC blood agar plate and precise 37$^0$C were also required in incubation step.

## 2.7 IDENTIFICATION OF CLINICAL BACTERIAL ISOLATES

Some clinical quinolone-resistant isolates from Malaysia and Thailand was identified by the use of API20∈ (Bio Mérieux SA, France) for those isolates of Enterobacteriaceae and other Gram-negative rods, API20N∈ (Bio Mérieux SA, France) for those of non-enteric Gram-negative rods.

## 2.8 GENETIC CHARACTERISATION OF MUTATION IN THE QRDR OF *GYRA* AND *PARC* GENES

### 2.8.1 Preparation of chromosomal DNA by enzyme lysis plus solvent extraction

Bacterial chromosomal DNA was separated from cells by using the modified protocol of Zyskind and Bernstein (1989) with no ether extraction step. A single colony was inoculated into 5 ml of appropriate nutrient broth and grown overnight in a $37^0$C incubator. A 1.2 ml volume of this culture was spun in a microcentrifuge (Microcentaur MSE) at 3000 rpm for about 15 seconds. The broth was discarded and the cell pellet resuspended in a volume of 0.31 ml HTE buffer (50 mM Tris-HCl, pH 8.0, 20 mM EDTA) by briefly vortexing to give no cell clumps and then 0.35 ml of 2% solution sarcosyl (*N*-lauroylsarcosine) was added with brief inversion of the tube, followed by 5 µl RNAase (10 mg/ml in TE buffer) (10mM Tris-HCl, pH8.0, 10 mM NaCl, 0.1 mM EDTA) and incubated at $37^0$C for 15 minutes. A volume of 35 µl of pronase solution (10 mg/ml pronase in 10 mM Tris-HCl pH 8.0, 10 mM NaCl, 0.1

mM EDTA buffer, 87$^0$C for 15 minutes to digest any DNases present) was added and the tubes incubated in a 50$^0$C waterbath until lysis had occurred (30-90 minutes). The DNA molecular weight was lowered to around 20 kb by vortexing the lysate for 2 minutes to shear the DNA. This decreased the viscosity and made it easier to extract the lysate with phenol:chloroform and ether. Three rounds of phenol:chloroform extraction were carried out by addition of an equal volume (0.70 ml) of phenol:chloroform 1:1, followed by brief vortex for few seconds. The samples were centrifuged at 13,000 rpm for 3 minutes and the top layer was carefully removed with a sterile Pasteur pipette and added to a new microcentrifuge tube. The white flocculent interphase must not be taken across and the phenol:chloroform waste was disposed of in an approved waste receptacle. The resulting lysate was treated with 70 µl of 0.3 M sodium acetate with good mixing, and an equal volume 0.7 ml of isopropanol. After adding the isopropanol, the tube was inverted five or six times. The sample was then frozen at -70$^0$C for 15 minutes. DNA precipitation was achieved after centrifugation at 13,000 rpm for 20 minutes and then the supernatant was poured off. The resulting pellet was washed three times by the addition of 70% ethanol, brief vortexing and centrifugation at 13,000 rpm for 5 minutes and then the supernatant was poured off. After being left to dry at room temperature, the dry pellet was resuspended in 50 µl of TE buffer, and stored at -20$^0$C for up to 1 month. The DNA suspension was ready to use as template for DNA amplification by PCR.

## 2.8.2 Preparation of chromosomal DNA by boiling

Another way used to prepare chromosomal DNA was by lysing cells in a hypotonic solution at high temperature ($100^0$C) usually in a boiling water bath. This was a quick and effective method of preparing DNA for PCR. The main limitation is that only a few cells usually $10^4$ or less, can be used; otherwise, the accumulation of cellular debris will begin to inhibit the reaction. This preparation was done by pelleting $10^2$-$10^4$ cells in a 15-ml conical tube in a benchtop centrifuge at 1200-1500 g for about 10 minutes. The cells were resuspended in 5 ml of phosphate-buffered saline and repelleted by centrifugation. (If necessary, this step was repeated to remove residual amounts of the original suspension buffer). The washed cells were resuspended again in 25-50 µl distilled water and transferred to a 0.5 microcentrifuge tube, followed by boiling in water for 3-5 minutes. The clear lysate was transferred to a new tube and was ready to use as template for PCR or kept at -$20^0$C for up to 1 month.

Another modified preparation was made by growing cells on an agar medium plate overnight and then suspending tiny amount of cells in 50-100 µl of sterile water and boiling for 3-5 minutes. The clear lysate was then ready for DNA amplification by PCR: 1 µl per 100 µl of PCR reaction; however, 3-5 µl when amplifying the QRDR of *Moraxella catarrhalis* or *Neisseria gonorrhoea.*

## 2.8.3 Verification of DNA Product by Agarose Gel Electrophoresis

DNA preparations were examined for their purity, quantity and size by conventional agarose gel elctrophoresis. Agarose gel electrophoretic analysis of DNA was performed with TAE (10X TAE pH7.6: 40 mM Tris-acetate, 1 mM EDTA) buffer. The gels (1% agarose) were made up by melting the appropriate amount of agarose (GibcoBRL, Montgomery, Maryland) in 1x TAE in a microwave oven. Gels were cast in an appropriate casting tray and, once set, placed in a gel electrophoresis tank (Bethesda Research Laboratories Horizon 20.25 gel tank, Life Technologies, Petersburg, Florida or a Pharmacia GNA-100 minitank) and immersed in 1X TAE buffer. Six μl of each PCR sample with 1 μl of tracking dye (30% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol) were loaded in a well at one end of the tray along with standard markers that were either "λ *Hind*III digest" (Sigma D-9780) or "100bp DNA ladder" (GibcoBRL, Montgomery, Maryland) for molecular weight reference. Electrophoresis at 100V was then performed for 20-30 minutes depending on the size of the DNA fragments concerned. After completion of electrophoresis, gels were stained in water containing 50 μg/L ethidium bromide for about half an hour. Samples were examined for their purity, quantity and size of bands by UV visualization on UV transillumination (UV Products, Cambridge). DNA preparations with good purity must show only one band. Their quantity and size can be estimated by comparison with that of standard DNA markers on the same gel.

## 2.8.4 DNA Amplification by Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) amplification of a specified fragment of DNA was carried out in a total volume of 100 or 50 μl prepared in a 0.5 ml polypropylene microcentrifuge tube (Alpha Laboratories) with a Techne PHC-2 Dri-Block Cycler (Cambridge, Cambs). The method used is based on Gibco basic PCR protocol and the hot start method in the protocol was used to ensure high specificity of the products synthesized. A volume of 100 μl PCR reaction consisted of the following components sitting on ice: ~10 ng DNA template (1-10 μl of the chromosomal DNA solution), 1x PCR buffer (GibcoBRL, Montgomery, Maryland), 1.5 mM $MgCl_2$ (GibcoBRL). 0.2 mM each dNTP (Boehringer, Mannheim), 0.5 μM of one biotin-labelled primer, and 1.5 μM of another primer without biotin (Oswel DNA Service Lab, University of Southampton), 2.5 μl of 1% W-1, 2-2.5 units of *Taq* DNA Polymerase (GibcoBRL, Montgomery, Maryland) and autoclaved distilled water to 100 μl. One positive and two negative controls were always run along with the samples to ensure that only the expected product was constructed. One negative control had water instead of two primers of the same volume; the other control had, instead of template, water to the same volume.

Four sets of primers were used; their sequences are shown below (Table 9) together with the gene and bacteria they were used for. All primer sets were used to amplify the region including QRDR.

**Table 9 Four sets of primers used in PCR to amplify *gyrA*'s and *parC*'s QRDR.**

| Name[1] | Sequence[2] | Reference | Region Amplified |
|---|---|---|---|
| 1a) *gyrA*-E-3' primer | ATGAGCGACCTTGCGAGAGAAATTACACCG | G6083 | *gyrA* |
| 1b) *gyrA*-E-5' primer | TTCCATCAGCCCTTCAATGCTGATGTCTTC* | G6085 | *gyrA* |
| 2a) *gyrA*-N-3' primer | CGCCATACCGACGGCGATACC | T1447 | *gyrA* |
| 2b) *gyrA*-N-5' primer | GACGGCCTAAAGCCGGTGCAC* | T1446 | *gyrA* |
| 3a) *parC*-E-3' primer | AGGTAGCATTTTCGGCTCCT* | V4640 | *parC* |
| 3b) *parC*-E-5' primer | AATGAGCGATATGGCAGAGC | V4641 | *parC* |
| 4a) *parC*-N-3' primer | TGAGCAACACCATAGGCAAG | V4643 | *parC* |
| 4b) *parC*-N-5' primer | TCCCATACCGATTCCAACAC* | V4642 | *parC* |

[1] Primer names with "E" is for *E. coli*, *Enterobacter sakazakii* and *Citrobacter freundii*; with "N" for *Neisseria gonorrhoeae* and *Moraxella* spp.

[2] Sequence with * is with 5-biotin labelled so subsequent strand separation could be performed with magnetic biotin-streptavidin separation (Dynabeads® M-280 Streptavidin). Primer set 1) and 3) was designed on inspection of the *gyrA* sequence of *E. coli* KL16 (Yoshida *et. al*, 1988); primer set 2) and 4), of *parC* sequence of *N. gonorrhoeae* (Belland *et. al*.,1994).

Reaction mixtures were overlaid with one or two drops of mineral oil to reduce evaporation, and at the tips of the tubes to improve thermal contact with the heating block. Thermal cycler condition used is shown below.

1) 96$^0$C for 30 seconds

2) 80$^0$C for until *Taq* DNA Polymerase was loaded

3) 96$^0$C for 15 seconds

4) 60$^0$C for 30 seconds (in case of *parC*, 53$^0$C for 1 min.)

5) 70$^0$C for 90 seconds (repeat step 3, 4 and 5 for 25-30 times)

6) 70$^0$C for 5 minutes

After completion of the PCR reaction, the biotin-labelled product were taken to a new tube without oil and placed on ice to quench any further reaction, or stored at -20$^0$C for up to 1 month for verification of PCR products by agarose gel electrophoresis and later DNA strand separation. Verification of PCR product by agarose gel electroporesis was done in the same way as for chromosomal DNA preparation in 2.8.3.

## 2.8.5 DNA-STRAND SEPARATION

The double-stranded PCR product with biotin labelled to one strand allows possible separation by magnetic biotin-streptavidin separation, which is accomplished by the use of Dynabeads®M-280 Streptavidin (Dynal A.S., Norway). The use of Dynabeads, as a magnetizable solid phase, for the capture and purification of the PCR product, allows simple, rapid and efficient preparation of immobilized single-stranded template for sequencing. The principle of the method is that end labelling of PCR product with biotin is achieved simply by using a biotinylated primer. Capture and purification are then accomplished by incubation with Dynabeads®M-280 Streptavidin and separation using a Dynal MPC (Magnetic Particle Concentrator). Once immobilized, the DNA is converted to a single-stranded template by elution with alkali and another magnetic separation. Immobilized single-stranded template prepared in this manner are suitable for manual and automated solid-phase DNA sequencing by the dideoxynucleotide chain termination method, where magnetic properties of the beads are used further to improve the quality of results. A 100% single-stranded template can be produced, without interference from primers, free nucleotides or the complementary strand. The benefit of the method is that PCR

reaction components (buffer, dNTP's) are removed at the same time as the single-stranded template is generated.

This DNA strand separation used in the course of this thesis followed the protocol recommended by Dynal with some changes. The modified method is shown below.

## 1. Preparation of Dynabeads

1.1 The Dynabeads®M-280 Streptavidin were resuspended by gently shaking the vial to obtain a homogeneous suspension.

1.2 An amount of 20 µl of the homogeneous Dynabeads for each template preparation was added to a 1 ml centrifuge tube.

1.3 The tube was placed in the Dynal MPC for at least 30 seconds so that all the beads were magnetically attracted to one small area of the tube. (The tube must not be removed during the separation process)

1.4 The supernatant was simply removed by aspiration with a pipette while the tube remained in the Dynal MPC. (The pipette tip must not touch the inside wall of the tube, where the Dynabeads were attracted to the magnet)

1.5 The tube was then removed from the Dynal MPC. The same amount as in step 1.2 of 2X concentrated B&W buffer (10mM Tris-HCl (pH 7.5), 1 mM EDTA and 2.0 M NaCl) was added along the inside of the tube where the Dynabeads were held. The beads were resuspended full but gently by pipette aspiration.

1.6 Steps 1.3, 1.4 and 1.5 were repeated twice.

## 2. Immobilization of the PCR product

2.1 Steps 1.3, 1.4 & 1.5 were repeated but the volume of 2X (concentrated) B&W was twice that in step 1.5.

2.2 To 40 µl of the amplified product, 40 µl of the washed beads were added.

2.3 The vial was incubated for 15 minutes at room temperature keeping the beads suspended by gentle rotation of the tube.

## 3. Melting the DNA duplex

3.1 The beads were washed by repeating steps 1.3, 1.4 and 1.5, and the pellet was resuspended in 40 µl/reaction of 2X B&W. (Note: The immobilized product could then be stored at $4^0$C for several weeks)

3.2 The beads were washed again and the pellet was resuspended in 8 µl of a freshly prepared 0.1 M NaOH. It was then incubated at room temperature for 10 minutes.

## 4. Separating the DNA strands

4.1 By using the Dynal MPC to collect the Dynabeads on the side of the tube, the NaOH supernatant containing the non-biotinylated DNA strands, was transferred to a clean tube for step 4.4.

4.2 The Dynabeads with the immobilized biotinylated DNA strands were washed once with 50 µl 0.1 M NaOH, once with 50 µl 1X B&W buffer, and once with 50 µl TE buffer.

4.3 The supernatant was removed and 13 µl of sterile MilliQ water was added. This final solution contained biotinylated DNA strands ready for sequencing.

4.4 To determine the nucleotide sequence of the complementary DNA template in the alkali supernatant, the NaOH supernatant from step 4.1 was added and mixed well with 4 µl 0.2 M HCl and immediately with 1 µl 1.0 M Tris-HCl (pH 7.5) without water added. This complementary DNA template was ready for sequencing.

Note: The same pipette was used for both NaOH and HCl, as small differences in calibration between different pipettes can cause neutralization problems. To save time and cost 0.1 M NaOH was prepared by diluting 1 M NaOH prepared in bulk which was stored in -70$^0$C up to 3 months.

## 2.8.6 DNA Sequencing

A 7 µl volume of the single-stranded DNA template either from step 4.3 or 4.4 in DNA-strand separation, was used routinely in the sequencing reaction which was carried out with a T7 Sequenase version 2.0 DNA Sequencing kit (US 70770) according to the manufacturer's protocol with [$^{35}$S] dATP (Amersham Life Sciences, UK) radiolabel and pyrophosphatase. Sequencing primers used were

the same as those used in Amplification of DNA by PCR. This sequencing is based on the chain-termination method to synthesis complementary DNA strands from single strand-DNA template amplified by PCR. When proper mixtures of dNTPs and one of the four ddNTPs were used, the DNA polymerase produced a fraction of the population of chains at each site where the ddNTP was incorporated. Four separate reactions, each with a different ddNTP gave the complete sequence information. A radioactively labelled nucleotide also was included in the synthesis, so the labelled DNA chains of various lengths were visualised by autoradiography after separation by high-resolution electrophoresis producing information of complementary sequence of the template. The protocol is as followed.

1. Annealing template and primer. Four ingredients were combined into a tube as followed:

| | | |
|---|---|---|
| Primer | 1 | µl (final concentration 0.5 pmol/µl) |
| DNA template (from step 4.3 or 4.4) | 7 | µl |
| T7 Sequenase reaction buffer | 2 | µl |
| Total | 10 | µl |

2. The capped tube was warmed to 65$^0$C for 2 minutes, then allowed to cool slowly to room temperature over a period of about 30 minutes. Then it was chilled on ice.

3. One µl T7 Sequenase enzyme was combined with 6.5 µl Enzyme Dilution Buffer and 0.5 µl pyrophosphatase.

4. Labelling reaction. The following were added to the annealed template-primer reaction (10 µl):

| | |
|---|---|
| DTT (0.1 M) | 1.0 µl |
| Labelling mix (diluted (1:5) | 2.0 µl |
| [$^{35}$S] dATP | 0.5 µl |
| T7 Sequenase DNA polymerase | 3.25 units (2 µl of 1:8 dilution) |

This mixture was mixed thoroughly and incubated for 3 minutes at room temperature or cooler (4-20$^0$C).

Note: [$^{35}$S] dATP should have specific activity of 1,000-1,200 Ci/mmol and the concentration about 10 µCi/µl.

5. Termination reactions.

5.1 Four tubes each labelled G, A, T and C were filled each with 2.5 µl of the appropriate dideoxy termination mix, and were then prewarmed to 37-45$^0$C (45$^0$C was preferable). It must not be below 37$^0$C.

5.2 When the labelling reaction was completed, 3.5 µl of it was transferred to each of the four pre-warmed tubes labelled G, A, T and C.

5.3 After 3 minute incubation at 37$^0$C, 4 µl of stop solution was added to each termination reaction, mixed and stored on ice. These terminated sequencing mixtures were ready for sequencing gel electrophoresis and were be stored at -20$^0$C for less than 7 days.

(Note: Except the enzyme, each of the kit components was thawn on ice, mixed by pumping and down several times with a micropipette. Whenever necessary,

the reaction tubes were briefly spun a microcentrifuge to collect the contents in the bottom of the tube.)

## 2.8.7 DNA Sequencing-Gel Electrophoresis and Autoradiography

DNA sequencing gel electrophoresis was performed with a Nucleic Acid Sequencing Cell (BioRad, Watford, Herts). The glass sequencing gel plates were thoroughly cleaned on one side each: once with water; twice with cleaning solution, twice with double distilled water, twice with ethanol; and three times with propanol or acetone (scrub hard and work a small area at a time on the second propanol/acetone wash). The cleaner the plates, the easier it was to pour a gel without bubbles. The two plates were then assembled together with 0.2 mm cleaned spacers separating the two plates. The top plate was siliconized with Gel Slick (AT Biochem, Malvern, Pennsylvania), in order to let the gel stick onto the unsiliconized glass plate when the apparatus was disassembled.

The best sequencing gel employed through the course was of 8%, which was prepared from 40% Accugel 19:1$^{TM}$(acrylamide:bis-acrylamide) gel solution (National Diaganostics, USA, Order No. EC-850, sequencing grade, gas stabilized). To make a sequencing gel 8 M urea and 1X TBE buffer were combined with 8% gel (19:1 = acrylamide:bis-acrylamide). It was heated briefly by heating in a microwave oven and then was swirled to dissolve urea in gel solution. The cool gel solution was then purified by millipore filtration. The gel solution was then ready for the plug: 30 ml of the gel solution was polymerised by the addition of 150 µl 25% ammonium

persulphate solution and 150 µl TEMED. Immediately, this was poured into the gel casting tray to form a plug. Next, the rest of the gel solution was cooled down to about $4^0$C as recommended by the manufacturer so that the solution is not set at the time of pouring. For each ml of remaining gel solution, 1 µl of 25% ammonium persulphate solution and 1 µl of TEMED was added and the solution poured carefully between the two plates of the gel apparatus. The flat edge of a shark toothed comb was pushed between the plates to layer the top of the gel. The gels should be prepare 2-20 hours prior to use. Once set, the comb was removed and the top of the gel was thoroughly flushed with distilled water. The sharktooth comb was then replaced with the points downwards just touching the surface of the gel. The gel was clamped into the sequencing apparatus and 1X TBE solution poured into the top and bottom reservoirs. The gel was then pre-run at 50 W for an hour and then at higher (70-100) wattage so that the gel temperature was at 55-$60^0$C. The gel was ready to be loaded with the extended sequencing mixtures. The samples were loaded in the order G, A, T and C immediately after denaturing the DNA by heating the samples to $85^0$C for 3 minutes. Immediately after this incubation, 2-3 µl of each sample were loaded onto the gel after the wells were rinsed out with buffer, being sure to rinse out all the urea-containing buffer from each well. The gel was then electrophoresed at $55^0$C and maintained at this temperature regardless of the wattage for 5-8 hours, depending on which part of the sequence was to be read. Once electrophoresis was complete, the glass plates were removed from the apparatus and the top plate very carefully removed. The bottom plate with the gel attached was then placed in a fixing bath containing 15% methanol and 5% acetic acid in water for 20

minutes. The plate and gel were then removed and a sheet of Whatman 3MM paper placed on top of the gel. Even pressure was then applied and the paper peeled off taking the gel with it. The gel attached to the paper was covered in Saran Wrap and dried in a vacuum gel drier for 2-3 hours at $80^0$C. When dry, the gel was placed in an autoradiography cassette (Amersham Life Sciences, UK) and exposed to Kodak Biomax MR-1 film at room temperature for about 10-20 hours after which the film was developed at the X-ray department, Royal Infirmary of Edinburgh.

# 3. INVESTIGATION OF CIPROFLOXACIN RESISTANCE IN CLINICAL BACTERIAL ISOLATES

## 3.1 INTRODUCTION

This section seeks to demonstrate whether there were any unusually high levels of ciprofloxacin (Cip) resistant bacteria amongst clinical isolates from Malaysia and Thailand. The results could lead to support the report of Turnidge (1995) that developing countries shows high antibiotic resistance and is lesser in countries with better welfare. If there was any unusually high level of resistance, their *gyrA* and *parC* genes responsible for high Cip resistance would be studied further. It was hoped that at the end of the study a solution to fluoroquinolone-resistance might be found. It was also decided to include *Moraxella catarrhalis* isolates from Edinburgh.

Urinary tract bacterial isolates from Malaysia (Kathiravel *et. al.*, (1994), Thailand and *Moraxella* isolates from Edinburgh were examined for their antibiotic sensitivity to Cip. If high Cip-resistant isolates were found they would be used for further study on *gyrA* or *parC* mutations, a common cause of Cip resistant mechanism.

Although, in general, fluoroquinolone resistance was the purpose of this investigation, Cip alone was used primarily as a representative because it was the most powerful fluoroquinolone. It is anticipated that resistance found to ciprofloxacin also commonly conferred potent to every other fluoroquinolones; however, despite this presumption, some other fluoroquinolones: ofloxacin,

norfloxacin and enoxacin, were also tested for their activities against some isolates (Table 13).

## 3.2 METHODS

Antibiotic sensitivity testing of Cip for those three groups of bacterial isolates was determined by double dilution-plate method (full details in 2.6) and their minimum inhibitory concentrations (MICs) were shown (Table 10). Some isolates with high resistance were then identified by API 20∈ or 20 N∈(full details in 2.7).

## 3.3 RESULTS

### 3.3.1 MALAYSIAN ISOLATES

Among 163 Malaysian urinary tract isolates collected by Kathiravel *et. al.*, (1994), 22% (36/163) had MIC of Cip ≥0.08 mg/L, and 6% ≥ 1 mg/L. The ten most resistant isolates were from four bacterial species: *E. coli, Acinetobacter baumanii, P. aeruginosa* and *Klebsiella* sp. Their MICs are displayed in Table 10. Some had remarkably high level of Cip resistantnce: two *E. coli*; 32, 128 mg/L and six *A. baumanii*, 4-128 mg/L. As *E. coli* is very common and widespread, it is regarded very serious and unusual that the two *E. coli* had such an unusually high resistance and so they were to be examined further in the next experiment. The rest of the Cip-resistant bacteria had intermediate resistance and they were *P. aeruginosa* KP174 (MIC of 2 mg/L) and *Klebsiella* sp. KP 50 (MIC of 4 mg/L).

**Table 10** **Minimum inhibitory concentrations (MIC) against Cip of the Malaysian clinical urinary-tract isolates.**

| COUNTRY | NAME | | MIC of Cip (mg/L) |
|---|---|---|---|
| Malaysia | *E. coli* | KP 88 | 128 |
| Malaysia | *E. coli* | KP 115 | 32 |
| Malaysia | *Acinetobacter baumanii* | KP 7 | 64 |
| Malaysia | *Acinetobacter baumanii* | KP 23 | 128 |
| Malaysia | *Acinetobacter baumanii* | KP 24 | 64 |
| Malaysia | *Acinetobacter baumanii* | KP 29 | 64 |
| Malaysia | *Acinetobacter baumanii* | KP 47 | 8 |
| Malaysia | *Acinetobacter baumanii* | KP 66 | 4 |
| Malaysia | *P. aeruginosa* | KP174 | 2 |
| Malaysia | *Klebsiella* sp. | KP 50 | 4 |
| Malaysia | *E. coli* | KP 42,62,90,126,147 | ≤0.031 |
| (reference strain) | *E. coli* | NCTC10418 | 0.016 |
| (reference strain) | *P. aeruginosa* | NCTC10662 | 0.12 |
| (reference strain) | *S. aureus* | NCTC 6571 | 0.12 |
| Malaysia | *Acinetobacter baumanii* | KP 31,32,33 | ≤0.031 |
| Malaysia | *P. aeruginosa* | KP 15,21,22 | ≤0.12 |
| Malaysia | *K. pneumoniae* | KP 3 & 5 | 0.06 |

### 3.3.2 THAILAND ISOLATES

Some urinary-tract bacteria were isolated from patients in Thailand and screened for high fluoroquinolone resistance. Thirteen isolates from Thailand were found to be highly resistant to Cip. After their antibiotic sensitivity to Cip was rechecked, all isolates were identified by API 20∈ or 20 N∈(full details in 2.7). Their identity and their MIC results are displayed in Table 11. Six isolates showed the same remarkably high MIC of 128 mg/L which was considered seriously high and it therefore implied possible failure in fluoroquinolone chemotherapy. Those six isolates were two *E. coli* (T281 & T295), three *Acinetobacter baumanii* (T76, T78 and T537) and one *Citrobacter freundii* (T1510).

The second highest value of MIC under this investigation was 32 mg/L shared by four isolates of *Enterobacter* spp.: *Ent. amnigenus* (T75), *Ent. cloacae* (T400 and T401) and *Ent. sakazakii* (T77). Some other isolates showing intermediate resistance were *Enterobacter aerogenes* (T296) and two *K. pneumoniae* (T70 & T410) with MIC of 4, 8 and 2 mg/L, respectively.

**Table 11** **Minimum inhibitory concentrations (MIC) against Cip of the Thai clinical urinary-tract isolates.**

| COUNTRY | NAME | | MIC of Cip (mg/L) |
|---|---|---|---|
| Thailand | *E. coli* | T 281 | 128 |
| Thailand | *E. coli* | T 295 | 128 |
| Thailand | *Acinetobacter baumanii* | T 76 | 128 |
| Thailand | *Acinetobacter baumanii* | T 78 | 128 |
| Thailand | *Acinetobacter baumanii* | T 537 | 128 |
| Thailand | *Enterobacter aerogenes* | T 296 | 4 |
| Thailand | *Enterobacter amnigenus* | T 75 | 32 |
| Thailand | *Enterobacter cloacae* | T 400 | 32 |
| Thailand | *Enterobacter cloacae* | T 401 | 32 |
| Thailand | *Enterobacter sakazakii* | T 77 | 32 |
| Thailand | *Citrobacter freundii* | T1510 | 128 |
| Thailand | *K. pneumoniae* | T 70 | 8 |
| Thailand | *K. pneumoniae* | T 410 | 2 |
| (reference strain) | *E. coli* | NCTC10418 | 0.016 |
| (reference strain) | *P. aeruginosa* | NCTC10662 | 0.12 |
| (reference strain) | *S. aureus* | NCTC 6571 | 0.12 |
| (reference strain) | *Enterobacter sakazakii* | NCTC 5920 | ≤0.06 |
| Malaysia | *Acinetobacter baumanii* | KP 31, 32, 33 | ≤0.03 |
| Malaysia | *K. pneumoniae* | KP 3 & 5 | 0.06 |
| Edinburgh | *Citrobacter freundii* | KP CitS | 0.012 |

### 3.3.3 *MORAXELLA* ISOLATES

A Cip resistant clinical isolate *Moraxella catarrhalis* KP4/R (Edinburgh) revealed an interesting MIC of 4 mg/L (Table 12), which is quite exceptional for this particular bacteria while sensitive strain KP6/S had an MIC of ≤0.03 mg/L. It was then to be investigated whether it had a *gyrA* or *parC* mutation as a mechanism of resistance. As *gyrA* and *parC* of this bacterial strain had never been sequenced before, PCR and sequencing primers were designed and based on *Neisseria gonorrhoeae* sequences.

**Table 12 Minimum inhibitory concentrations (MIC) against Cip of *Moraxella catarrhalis* and *Neisseria gonorrhoeae.***

| COUNTRY | NAME | | MIC of Cip (mg/L) |
|---|---|---|---|
| Edinburgh | *M. catarrhalis* | KP 4/R | 4.0 |
| Edinburgh | *M. catarrhalis* | KP 6/S | ≤0.03 |
| Edinburgh | *N. gonorrhoea* | KP14240 | 0.008 |
| Edinburgh | *N. gonorrhoea* | KP13843 | 0.004 |
| Edinburgh | *N. gonorrhoea* | KP31267 | ≤0.001 |

## 3.4 DISCUSSION

In 1990, a group of researchers stated that high-level resistance to fluoroquinolones (i.e. MIC of ciprofloxacin, >1 mg/L) is rarely found among clinical isolates of *E. coli* (Barry *et. al.*, 1990); however, >6% of clinical *E. coli* with Cip MIC of 1 mg/L was found in Malaysia in 1994. Ciprofloxacin resistance among clinical isolates from Malaysia and Thailand were high to seriously high, especially *E. coli* (MIC of 32 to

128 mg/L). As so far reported, the highest level of MIC against Cip for clinical *E. coli* was 128 mg/L (Vila *et. al.*, 1994), the same as found here. For *Acinetobacter baumannii*, 64 mg/L (Bajaksouzian *et. al.*, 1997) but 128 mg/L found here; and for *Enterobacter* spp. and *Citrobacter freundii*, it seemed that there was no reports on clinical high Cip resistance. This leads to concern of how they became so heavily resistant.

Accordingly, some resistant isolates, along with sensitive bacteria of the same species, were chosen for further study on mutation mechanism in *gyrA* and *parC*. The chosen isolates were *E. coli* KP 88 and KP115 from Malaysia and T281 and T295 from Thailand; together with two isolates whose *gyrA* and *parC* sequence had never been sequenced: *Citrobacter freundii* T 410 (MIC of 128 mg/L) and *Enterobacter sakazakii* T 77 (MIC of 32 mg/L). Also, isolate *Moraxella catarrhalis* KP4/R was included for furthur study.

As several unusually high Cip resistant isolates were found, it supports the idea of Turnidge (1995) that fluoroquinolone resistant rates are high in the developing countries like Thailand and moderate in transitional countries like Malaysia.

Ofloxacin, norfloxacin and enoxacin were also tested for their activities against some clinical isolates together with ciprofloxcin (Table 13).

**Table 13** **Minimum inhibitory concentrations (MIC) against ciprofloxacin, ofloxacin, norfloxacin and enoxacin of some Malaysian clinical urinary-tract isolates and *Moraxella catarrhalis* isolated in Edinburgh.**

| COUNTRY | NAME | MIC (mg/L) | | | |
|---|---|---|---|---|---|
| | | Cip | Ofx | Nfx | Eno |
| (reference strain) | *E. coli* NCTC10418 | 0.016 | 0.5 | ND | ND |
| (reference strain) | *P. aeruginosa* NCTC10662 | 0.12 | 2 | 1 | 1 |
| (reference strain) | *S. aureus* NCTC 6571 | 0.12 | 0.5 | 1 | 1 |
| Edinburgh | *M. catarrhalis* KP 4/R | 4 | ND | ND | ND |
| Edinburgh | *M. catarrhalis* KP 6/S | ≤0.032 | 8 | 16 | 16 |
| Malaysia | *A. baumanii* KP 7 | 64 | 16 | >128 | >128 |
| Malaysia | *A. baumanii* KP 23 | 128 | 16 | >128 | >128 |
| Malaysia | *A. baumanii* KP 24 | 64 | 16 | >128 | >128 |
| Malaysia | *A. baumanii* KP 29 | 64 | 16 | >128 | >128 |
| Malaysia | *A. baumanii* KP 47 | 8 | 4 | ND | ND |
| Malaysia | *Klebsiella* sp. KP 50 | 4 | 4 | ND | ND |
| Malaysia | *A. baumanii* KP 66 | 4 | 8 | ND | ND |
| Malaysia | *E. coli* KP 88 | 128 | 128 | >128 | >128 |
| Malaysia | *E. coli* KP115 | 32 | 32 | 32 | 128 |

Note: ND indicates "not determined".

# 4. GENETIC CHARACTERISATION OF MUTATION IN THE QRDR OF *GYRA* AND *PARC* GENES OF CIP RESISTANT BACTERIA OF CLINICAL ISOLATES

# 4.1 INTRODUCTION

The occurrence of clinical isolates highly resistant to fluoroquinolones has been reported recently. Bacterial resistance to fluoroquinolones drugs has been frequently shown to occur concurrently with mutations in either or both *gyrA* (Chen *et. al.*, 1996; Gellert *et. al.*, 1977; Hane & Wood, 1969; Heisig, 1996; Nakamura *et. al.*, 1989; Reece & Maxwell, 1991; Sugino *et. al.*, 1977; Yoshida *et. al.*, 1988, 1990a) and *parC* (Chen *et. al.*, 1996; Gootz *et. al.*, 1996; Heisig, 1996).

By far the most commonly reported type of mechanism causing highly decreased susceptibility to fluoroquinolone is a mutation in the specific sequence called quinolone resistance-determining region (QRDR) (Yoshida *et. al.*, 1990b) of *gyrA* gene and, more recently, *parC* gene (Chen *et. al.*, 1996; Heisig, 1996). Such mutations are correlated to a change in MICs of a cell compared to a sensitive one. The QRDR spans the area of the protein between alanine-67 and glutamine-106 of *E. coli* (Swanberg & Wang, 1987; Yoshida *et. al.*,1990a; Hallett & Maxwell, 1991).

As point mutations in QRDR were commonly found to be a main mechanism of bacteria to resist fluoroquinolone activity, the following mutation analysis was therefore designed to establish if unusually high resistance in the Malaysian and Thailand isolates was associated with any mutations in QRDR of *gyrA* and *parC*.

In order to obtain the QRDR sequences for mutation analysis, amplification of the QRDR sequence of each isolate must be obtained and this was achieved mainly by cell-directed polymerase chain reaction (PCR) technique. The PCR fragments were

sequenced afterwards and finally analysed for mutations with the aim of identifying any differences that were compatible with the unusually high resistance. The result may provide useful information to best manage resistance in clinical bacteria.

Table 14 below shows all clinical bacterial isolates studied; 1 to 7 with unusually high Cip resistance, 8 to 13 as sensitive controls.

**Table 14 All Cip resistant bacteria of clinical isolates from Malaysia and Thailand and Cip sensitive controls.**

| NAME | MIC of Cip (mg/L) | COUNTRY |
|---|---|---|
| 1) *E. coli* KP 115 | 32 | Malaysia |
| 2) *E. coli* KP88 | 128 | Malaysia |
| 3) *E. coli* T281 | 128 | Thailand |
| 4) *E. coli* T295 | 128 | Thailand |
| 5) *Enterobacter sakazakii* T77 | 32 | Thailand |
| 6) *Citrobacter freundii* T1510 | 128 | Thailand |
| 7) *Moraxella catarrhalis* KP 4/R | 4.0 | Edinburgh |
| 8) *E. coli* KP 42, 62, 90, 111, 126, &147 | $\leq$0.031 | Malaysia |
| 9) *Ent. sakazakii* NCTC5920 | $\leq$0.06 | (reference strain) |
| 10) *C. freundii* KP CitS | 0.012 | Edinburgh |
| 11) *M. catarrhalis* KP 6/S | $\leq$0.03 | Edinburgh |
| 12) *N. gonorrhoeae* KP31267 | $\leq$0.001 | Edinburgh |
| 13) *N. gonorrhoeae* KP13843 | 0.004 | Edinburgh |
| 14) *N. gonorrhoeae* KP14240 | 0.008 | Edinburgh |

# 4.2 METHODS

In order to get sequence information for mutation analysis, a number of experiments were set up as followed:

4.2.1 Preparation of chromosomal DNA

4.3.1 DNA amplification by polymerase chain reaction (PCR)

4.4.1 Verification of PCR product by agarose gel electrophoresis

4.5.1 DNA-strand separation

4.6.1 DNA sequencing

4.7.1 DNA sequencing gel electrophoresis

4.8.1 Mutation analysis

## 4.2.1 METHOD: PREPARATION OF CHROMOSOMAL DNA

This method was to separate chromosomal DNA from cells of interest so that the preparations were suitable for amplification by PCR technique. This was done by two methods: (1) 'enzyme lysis plus solvent extraction' and (2) boiling. The former was designed by Zyskind and Bernsterin (1989), with a slight modification (full details in 2.8.1.) The latter method was by simple lysing cells in a hypotonic solution at high temperature ($100^0$C) (full details in 2.8.2). Chromosomal preparation by boiling is effective and simpler than the former method. However, the disadvantage of the boiling method is that there is no appropriate way to verify the result, which can be assumed after polymerase chain reaction result obtained.

Chromosomal DNA preparations obtained by 'enzyme lysis plus solvent extraction' were subsequently examined by agarose gel electrophoresis (full details in 2.8.3).

### 4.2.2 RESULT AND DISCUSSION

A uniform, single strong band of expected chromosomal DNA size on agarose gel was produced from all 20 clinical bacteria. Figure 6 displays some representatives chromosomal DNA bands. All bacteria gave bands of the same type and size. Although each DNA concentration was not precisely the same as indicated by intensity and thickness of bands, all DNA preparations were good enough to act as DNA templates for PCR.

The modified method employed here for chromosomal DNA preparations proved to be successful in producing fine chromosomal DNA for amplification by PCR technique. Those DNA with high purity, correct size and appropriate quantity were therefore to be amplified by PCR technique, as well as those chromosomal DNA preparations prepared by boiling.

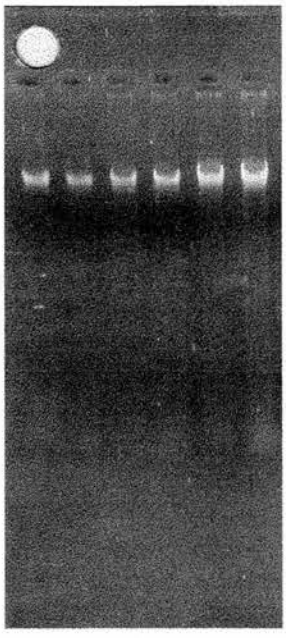

**Figure 6** **UV illuminated chromosomal DNA bands prepared by 'enzyme lysis plus solvent extraction' on agarose gel.**

### 4.3.1 METHOD: DNA AMPLIFICATION BY POLYMERASE CHAIN REACTION (PCR)

The objective was to amplify the QRDR segment of *gyrA* and *parC* genes of the 20 clinical bacteria in order to sequence the QRDR. Chromosomal DNA produced by both 'enzyme lysis plus solvent extraction' and boiling (4.2.1) were used as template to be hybridized by a set of primers for *gyrA* and for *parC* QRDR (full details in 2.8.4). The use of biotinylated primers (Table 1) provided the end labelling of PCR product with biotin, which was needed in single-stranded DNA separation (4.4.1) The PCR products were then verified on 1% agarose gel electrophoresis (full details in 2.8.3).

In case of *N. gonorrhoeae* and *M. catarrhalis*, 10% glycerol were combined in PCR reactions to improve the specificity and yield of PCR products; if this step was not included multiple and non specific products were found.

### 4.3.2 RESULT AND DISCUSSION

PCR products were examined on 1% agarose gel (2.8.3) with standard DNA markers "100bp DNA ladder" (GibcoBRL, Montgomery County, Maryland). Bacteria in Enterobacteriaceae was expected to produce 630 bp of *gyrA* QRDR and 475 bp of *parC* QRDR, whereas those in Neisseriaceae were expected to produce 423 and 496 bp of *gyrA* and *parC* QRDR, respectively.

Cell-directed PCR technique worked well and made the step of chromosomal DNA preparation (4.2.1) unnecessary.

When producing *parC* QRDR, PCR products of expected sizes were achieved from all the clinical bacteria, except from both *M. catarrhalis* KP4/R and KP6/S, which produced multiple bands of non-expected sizes. However, three *Neisseria gonorrhoeae* KP14240, KP13843 & KP31267 as *Moraxella*'s positive control gave good PCR bands of corrected size. Figure 7 displays representative bands of PCR products of those clinical bacteria.

The cause of being unable to produce good PCR products of *parC* QRDRs of both *M. catarrhalis* could be that the sequences of the primers and the bacterium do not perfectly match because the primers had been designed and based on *N. gonorrhoeae* sequences. It might be possible that some nucleotides of *M. catarrhalis*'s equivalent

sequences were not complementary to that of the primers, which was likely to prime to other sites with more or less complementary sequence.

With both *M. catarrhalis*, the same overall result was obtained even though different conditions of PCR had been set up, i.e. different variables were devised: $MgCl_2$ concentration (2, 3, 4.5 and 5 mM), annealing temperature (50, 55, 60, 65 and $70^0$C), addition of some additives (DMSO (5% final concentration) and glycerol (10% final concentration). [The additives added were to promote polymerease stability and processivity or increase hybridisation stringency, and strategies that reduce nonspecific primer-template interactions.]

All the 38 PCR products obtained, of correct sizes, were next separated into two single strand-DNA molecules for further sequencing.

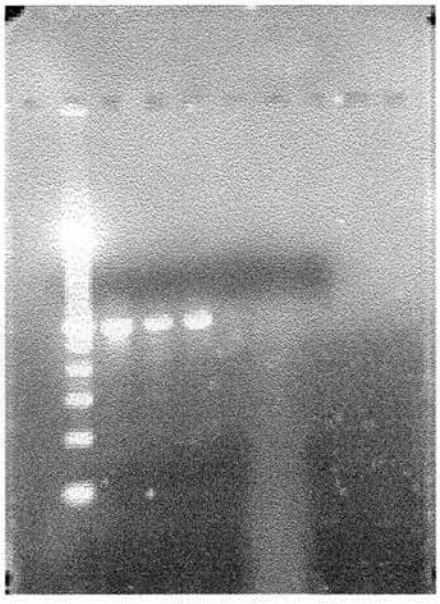

**Figure 7** **UV illuminated PCR products of *E. coli*'s *gyrA* QRDRs on agarose gel.**

## 4.4.1 METHOD: DNA-STRAND SEPARATION

This experiment was to separate PCR products, which are double-stranded DNA into single-stranded DNA molecules so that they are ready to be sequenced. The separation was accomplished by making use of Dynabeads®M-280 Streptavidin (Dynal A.S., Norway), which is based on a magnetic biotin-streptavidin separating technique (full details in 2.8.5). The use of Dynabeads, as a magnetizable solid phase, for the capture and purification of PCR products, allows simple preparation of immobilized single-stranded template, and therefore eliminates centrifugation steps and simplifies liquid handling (no need for ethanol or phenol extraction).

There were 40 double-stranded DNA samples from 20 clinical bacteria to be separated- no PCR products of *parC* from two of *M. catarrhalis* were achieved.

### 4.4.2 RESULT AND DISCUSSION

All the PCR products were successfully separated into two single-stranded DNA molecules (This was proved by the subsequently sequencing results). These both complementary DNA strands were sequenced, as double checks, to assure correct and reproducible sequencing results.

Note: These single-stranded DNA preparations can be stored in -20$^0$C up to two weeks. Precise concentrations of chemicals are critical, especially 0.1 M NaOH and 0.2 M HCl.

### 4.5.1 METHOD: DNA SEQUENCING

Good results can only be obtained when there is not a mistake through the course of the sequencing experiment, especially in the time in termination reaction and in the degree of temperature. Seventy-two single-stranded DNA molecules were manually sequenced using radiolabel [$^{35}$S] dATP (Amersham Life Sciences, UK) with a T7 Sequenase version 2.0 DNA Sequencing kit (US 70770) (full details with some modifications in 2.8.6).

For DNA amplified with the biotinylated version of the reverse PCR primer and the nonbiotinylated version of the forward PCR primer, the forward sequencing primer

must be used for sequencing the immobilized DNA template. The reverse sequencing primer must be used for sequencing the eluted DNA template.

All the samples were from 20 clinical bacteria and the regions to be sequenced were of *gyrA* and *parC* QRDR. As recommended, each samples were sequenced at least twice separately to ensure reproducible results.

## 4.5.2 RESULT AND DISCUSSION

At the end all the terminated sequencing mixtures were achieved and ready for next step of this analysis. The mixtures were able to be kept at -20$^0$C for up to 7 days.

It is recommended that, after completed extension in sequencing reaction, excess sequencing primers and unincorporated nucleotides in the supernatant are removed by another magnetic separation. However; when the magnetic separation was not readily observed, fair sequencing results were still be obtained in this study.

## 4.6.1 DNA SEQUENCING–GEL ELECTROPHORESIS

After the extended sequencing-mixture samples were denatured by heating to 85$^0$C for 3 minutes, they were loaded onto 6% sequencing gels (17 inches x 14 inches x 0.2 mm) and run at gel temperature of 55$^0$C, regardless of the wattage, for 5-8 hours. Once electrophoresis was complete, the gel with the bottom glass plate was submerged in a fixing solution (15% methanol and 5% acetic acid in water) for 20 minutes to wash off the urea. The gel was then dried and autoradiographed onto an X-ray film, which was developed 10-20 hours later. The sequencing bands on the

film were read, compared and analysed. The material and methods used are described in full in 2.8.7.

### 4.6.2 RESULT AND DISCUSSION

All the *gyrA* and *parC* QRDR sequences were achieved and the results were shown below, except *parC* QRDR sequences of both sensitive and resistant *M. catarrhalis*, which were not accomplished owing to the lack of complementation between primers and expected sites in *parC* region producing multiple DNA products of incorrect size.

## 4.6.2.1 RESULT: GYRA QRDR SEQUENCE OF ENTEROBACTERIACEAE

A 630-base region of partial *gyrA* gene was sequenced and amino acid residues at position 17 to 166 including QRDR (amino acid residues at 67-106) within brackets are shown below. The codons 83 and 87 are underlined. From 2) to 8) red capitals in resistant sequence indicate nucleotides and amino acids different from that of ECGYRA or its control and sensitive sequence and only section where nucleotide changes occurred is shown. (The numbers given along with all the sequences are based on nucleotide numbers of *gyrA* gene of *E. coli* ECGYRA.)

1) Standard Cip sensitive-*gyrA* gene of *E. coli* ECGYRA, as listed in GENBANK (X06744), with MIC of <0.031 mg/L (Yoshida *et. al.* (1988).

```
231 g ctg aag agc tcc tat ctg gat tat gcg atg tcg gtc att gtt ggc
      L   K   S   S   Y   L   D   Y   A   M   S   V   I   V   G
     17
277   cgt gcg ctg cca gat gtc cga gat ggc ctg aag ccg gta cac cgt
      R   A   L   P   D   V   R   D   G   L   K   P   V   H   R

333   cgc gta ctt tac gcc atg aac gta cta ggc aat gac tgg aac aaa
      R   V   L   Y   A   M   N   V   L   G   N   D   W   N   K

367   gcc tat aaa aaa tct[gcc cgt gtc gtt ggt gac gta atc ggt aaa
      A   Y   K   K   S   A   R   V   V   G   D   V   I   G   K
                        67
412   tac cat ccc cat ggt gac tcg gcg gtc tat gac acg atc gtc cgc
      Y   H   P   H   G   D   S   A   V   Y   D   T   I   V   R
                            83              87
457   atg gcg cag cca ttc tcg ctg cgt tat atg ctg gta gac ggt cag]
      M   A   Q   P   F   S   L   R   Y   M   L   V   D   G   Q
                                                          106
502   ggt aac ttc ggt tct atc gac ggc gac tct gcg gcg gca atg cgt
      G   N   F   G   S   I   D   G   D   S   A   A   A   M   R

547   tat acg gaa atc cgt ctg gcg aaa att gcc cat gaa ctg atg gcc
      Y   T   E   I   R   L   A   K   I   A   H   E   L   M   A

592   gat ctc gaa aaa gag acg gtc gat ttc gtt gat aac tat gac ggc
      D   L   E   K   E   T   V   D   F   V   D   N   Y   D   G

637   acg gaa aaa att ccg gac gtc atg cca acc aaa att cct aac ctg 681
      T   E   K   I   P   D   V   M   P   T   K   I   P   N   L
                                                          166
```

2) Six Cip sensitive-*gyrA* gene of *E. coli* KP 42, 62, 90, 111, 126 and 147 (Malaysia) with MIC of <0.031 mg/L.

- Altogether only 4 nucleotides were changed resulting in no amino-acid mutation.

```
412   tac cat ccc cat ggt gac tcg gcg gtT tat gac acg atc gtc cgT
       Y   H   P   H   G   D   S   A   V   Y   D   T   I   V   R
                               83                  87
457   atg gcg cag cca ttc tcg ctg cgt taC atg ctg gta gac ggt cag]
       M   A   Q   P   F   S   L   R   Y   M   L   V   D   G   Q
                                                               106
502   ggt aac ttc ggt tcC atc gac ggc gac tct gcg gcg gca atg cgt
       G   N   F   G   S   I   D   G   D   S   A   A   A   M   R
```

3) Cip resistant-*gyrA* gene of *E. coli* isolate KP115 (Malaysia) with an MIC of 32 mg/L.

- Five nucleotides within QRDR were changed resulting in 2 amino-acid mutations:

  Ser-83-Leu and Asp-87-Asn.

- Two nucleotide outside QRDR were changed; no amino-acid mutation.

```
412   tac cat ccc cat ggt gac tTg gcg gtT tat Aac acg atc gtc cgT
       Y   H   P   H   G   D   L   A   V   Y   N   T   I   V   R
                               83                  87
457   atg gcg cag cca ttc tcg ctg cgt taC atg ctg gta gac ggt cag]
       M   A   Q   P   F   S   L   R   Y   M   L   V   D   G   Q
                                                               106
502   ggt aac ttc ggt tcC atc gac ggc gac tct gcg gcg gca atg cgt
       G   N   F   G   S   I   D   G   D   S   A   A   A   M   R

547   tat acg gaa atc cgt ctg gcg aaa att gcc cat gaa ctg atg gcc
       Y   T   E   I   R   L   A   K   I   A   H   E   L   M   A

592   gat ctc gaa aaa gag acg gtc gat ttc gtt gat aac tat gac ggc
       D   L   E   K   E   T   V   D   F   V   D   N   Y   D   G

637   acg gaa aaa att ccC gac gtc atg cca acc aaa att cct aac ctg
       T   E   K   I   P   D   V   M   P   T   K   I   P   N   L
```

4) Cip resistant-*gyrA* gene of *E. coli* isolate KP88 (Malaysia), T281 & T295 (Thailand); all with MICs of 128 mg/L.

- Five nucleotides within QRDR were changed resulting in 2 amino-acid mutations: Ser-83-Leu and Asp-87-Tyr.
- Three nucleotides outside QRDR were changed; no amino-acid mutation.

```
412   tac cat ccc cat ggt gac tTg gcg gtT tat Tac acg atc gtc cgT
       Y   H   P   H   G   D   L   A   V   Y   Y   T   I   V   R
                               83                  87
457   atg gcg cag cca ttc tcg ctg cgt taC atg ctg gta gac ggt cag]
       M   A   Q   P   F   S   L   R   Y   M   L   V   D   G   Q
                                                                106
502   ggt aac ttc ggt tcC atc gac ggc gac tct gcg gcg gca atg cgt
       G   N   F   G   S   I   D   G   D   S   A   A   A   M   R

547   tat acg gaa atc cgt ctg gcg aaa att gcc cat gaa ctg atg gcc
       Y   T   E   I   R   L   A   K   I   A   H   E   L   M   A

592   gat ctc gaa aaa gag acg gtc gat ttc gtt gat aac tat gac ggc
       D   L   E   K   E   T   V   D   F   V   D   N   Y   D   G

637   acg gaa aaa att ccT gac gtc atg ccG acc aaa att cct aac ctg
       T   E   K   I   P   D   V   M   P   T   K   I   P   N   L
```

5) Cip sensitive-*gyrA* gene of *C. freundii* CitS (Edinburgh) with MIC of 0.012 mg/L.

In comparison to *E. coli* ECGYRA (1):

- 16 nucleotides within QRDR were changed resulting in 2 amino acids different:

  Ser-83-Thr and Ile-112-Val.

- 29 nucleotides outside QRDR were changed; 3 amino acids different:

  Leu-127-Met, Ala-128-Ser and Ile-155-Lys.

```
231 g ctg aag agc tcA tat ctg gat tat gcg atg tcg gtc att gtt ggc
      L   K   S   S   Y   L   D   Y   A   M   S   V   I   V   G
     17
277   cgt gcg ctg cca gaC gtc cga gat ggc ctg aaA ccg gtT cac cgt
      R   A   L   P   D   V   R   D   G   L   K   P   V   H   R

333   cgc gta ctt tac gcc atg aac gta TtG ggc aaC gac tgg aaT aaa
      R   V   L   Y   A   M   N   V   L   G   N   D   W   N   K

367   gcc tat aaa aaa tct[gcc cgt gtc gtt ggt gac gta atc ggt aaa
      A   Y   K   K   S   A   R   V   V   G   D   V   I   G   K
                            67
412   tac caC ccT cat ggt gaT AcC gcC gtT taC gac acC atT gtT cgT
      Y   H   P   H   G   D   T   A   V   Y   D   T   I   V   R
                          83              87
457   atg gcg cag cca ttc tcC Ttg cgt taC atg ctg gta gaT ggt cag]
      M   A   Q   P   F   S   L   R   Y   M   L   V   D   G   Q
                                                          106
502   ggt aac ttT ggt tct Gtc gaT ggc gac tcC gcA gcg gcG atg cgt
      G   N   F   G   S   V   D   G   D   S   A   A   A   M   R
                      112
547   tat acg gaa atc cgt Atg Tcg aaa atC gcc cat gaG ctg atg gcT
      Y   T   E   I   R   M   S   K   I   A   H   E   L   M   A
                      127 128
592   gaC ctG gaa aaa gaA acg gtT gat ttc gtC gat aac taC gac ggc
      D   L   E   K   E   T   V   D   F   V   D   N   Y   D   G

637   acC gaa aaa aAA ccT gac gtc atg cca acc aaa att cct aac ctg
      T   E   K   K   P   D   V   M   P   T   K   I   P   N   L
                155                                         166
```

6) Cip resistant-*gyrA* gene of *C. freundii* isolate T1510 (Thailand) with MIC of 128 mg/L. In comparison to the sensitive control (5):

- 2 nucleotides within QRDR were changed resulting in 2 amino-acid mutations: Thr-83-Ile and Asp-87-Tyr.
- 2 nucleotides outside QRDR were changed; no amino-acid mutation.

```
412   tac cac cct cat ggt gat aTc gcc gtt tac Tac acc att gtt cgt
       Y   H   P   H   G   D   I   A   V   Y   Y   T   I   V   R
                               83                  87
457   atg gcg cag cca ttc tcc ttg cgt tac atg ctg gta gat ggt cag]
       M   A   Q   P   F   S   L   R   Y   M   L   V   D   G   Q
                                                               106
502   ggt aac ttt ggt tct gtc gat ggc gac tcc gca gcg gcg atg cgt
       G   N   F   G   S   V   D   G   D   S   A   A   A   M   R

547   tat acg gaa atc cgt atg tcg aaa atc gcc cat gag ctg atg gct
       Y   T   E   I   R   M   S   K   I   A   H   E   L   M   A

592   gac ctg gaa aaa gaa acg gtt gat ttc gtc gat aac taT gac ggc
       D   L   E   K   E   T   V   D   F   V   D   N   Y   D   G

637   acG gaa aaa aaa cct gac gtc atg cca acc aaa att cct aac ctg
       T   E   K   K   P   D   V   M   P   T   K   I   P   N   L
```

7) Cip sensitive-*gyrA* gene of *Enterobacter sakazakii* NCTC5920 with MIC of ≤0.06 mg/L. In comparison to *E. coli* ECGYRA (1):

- 15 nucleotides within QRDR were changed resulting in no amino acid different.
- 30 nucleotides outside QRDR were changed; no amino acid different.

```
231 g ctg aaA agc tcc tat ctg gaC tat gcg atg tcg gtc att gtt ggc
      L   K   S   S   Y   L   D   Y   A   M   S   V   I   V   G

277   cgt gcg ctT ccG gat gtc cga gat ggc ctC aaA ccg gta cac cgt
      R   A   L   P   D   V   R   D   G   L   K   P   V   H   R

333   cgc gta ctt tac gcc atg aac gtG TtG ggc aat gac tgg aaT aaa
      R   V   L   Y   A   M   N   V   L   G   N   D   W   N   K

367   gcc taC aaa aaa tcC[gcc cgt gtc gtt ggt gac gta atc ggt aaa
      A   Y   K   K   S   A   R   V   V   G   D   V   I   G   K
                          67
412   tac cat ccc caC ggt gaT tcC gcC gtc taC gaT acC atT gtA cgT
      Y   H   P   H   G   D   S   A   V   Y   D   T   I   V   R
                              83              87
457   atg gcT cag ccG ttc tcg ctg cgC tat atg ctg gtG gaT ggt cag]
      M   A   Q   P   F   S   L   R   Y   M   L   V   D   G   Q
                                                          106
502   ggC aac ttc ggt tct atc gac ggc gac tcC gcC gcg gcG atg cgt
      G   N   F   G   S   I   D   G   D   S   A   A   A   M   R

547   tat acg gaa atc cgt ctg gcg aaG atC gcc cat gaa ctg atg gcc
      Y   T   E   I   R   L   A   K   I   A   H   E   L   M   A

592   gaC ctc gaa aaa gaA acC gtT gat ttT gtC gat aac tat gac ggc
      D   L   E   K   E   T   V   D   F   V   D   N   Y   D   G

637   acg gaa aaG atC ccg gac gtc atg ccG acc aaa atC ccG aaT ctg
      T   E   K   I   P   D   V   M   P   T   K   I   P   N   L
```

8) Cip resistant-*gyrA* gene of *Enterobacter sakazakii* T77 (Thailand) with MIC of 32 mg/L. In comparison to the sensitive control (7):

- 13 nucleotides within QRDR were changed resulting in 2 amino-acid mutations: Ser-83-Tyr, Asp-87-Asn.
- 29 nucleotides outside QRDR were changed; no amino-acid mutation.

```
231 g ctg aaa agc tcc tat ctg gac tat gcg atg tcg gtc att gtt ggc
      L   K   S   S   Y   L   D   Y   A   M   S   V   I   V   G

277   cgt gcg ctG ccg gaC gtc cgC gat ggc ctG aaG ccg gta cac cgt
      R   A   L   P   D   V   R   D   G   L   K   P   V   H   R

333   cgc gta ctA tac gcc atg aac gtA ttg ggc aat gac tgg aat aaa
      R   V   L   Y   A   M   N   V   L   G   N   D   W   N   K

367   gcc tac aaa aaa tcT[gcc cgt gtc gtt ggt gac gta atc ggt aaa
      A   Y   K   K   S   A   R   V   V   G   D   V   I   G   K
                        67
412   tac cat ccT caT ggt gat tAc gcG gtG tac AaC acc att gtC cgt
      Y   H   P   H   G   D   Y   A   V   Y   N   T   I   V   R
                            83                  87
457   atg gcG cag ccA ttc tcg ctg cgT taC atg ctg gtA gat ggt cag]
      M   A   Q   P   F   S   L   R   Y   M   L   V   D   G   Q
                                                            106
502   ggT aac ttT ggt tct atc gac ggc gac tcc gcc gcg gcA atg cgt
      G   N   F   G   S   I   D   G   D   S   A   A   A   M   R

547   tat acg gaa atc cgt ctg gcg aag atT gcc cat gaG ctg atg gcT
      Y   T   E   I   R   L   A   K   I   A   H   E   L   M   A

592   gac ctG gaa aaa gaG acG gtt gat ttC gtT gat aac taC gac ggc
      D   L   E   K   E   T   V   D   F   V   D   N   Y   D   G

637   acg gaa aaA atT ccT gac gtT atg ccA acG aaG atc ccA aaC ctg
      T   E   K   I   P   D   V   M   P   T   K   I   P   N   L
```

Results of the association of sequence changes with Cip MIC are shown in Table 15 below.

**Table 15 A summary of the association of QRDR alterations in *gyrA* of *E. coli*, *C. freundii* and *Ent. sakazakii* with Cip MICs are shown below.**

| NAME and COUNTRY | | Amino Acid Number 83 | 87 | MIC (mg/L) of Cip |
|---|---|---|---|---|
| 1) Sensitive *E. coli* ECGYRA, | GENBANK | Ser (S) | Asp (D) | ≤0.031 |
| 2) Sensitive *E. coli* (6 isolates), | Malaysia | Ser (S) | Asp (D) | ≤0.031 |
| 3) Resistant *E. coli* KP115, | Malaysia | Leu (L) | Asn (N) | 32 |
| 4) Resistant *E. coli* KP88, T281 & T295, | Malaysia Malaysia | Leu (L) | Tyr (Y) | 128 |
| 5) Sensitive *C. freundii* CitS | Edinburgh | Thr (T) | Asp (D) | 0.012 |
| 6) Resistant *C. freundii* T1510, | Thailand | Ile (I) | Tyr (Y) | 128 |
| 7) Sensitive *Ent. sakazakii* NCTC5920, | NCTC | Ser (S) | Asp (D) | ≤0.06 |
| 8) Resistant *Ent. sakazakii* T77, | Thailand | Tyr (Y) | Asn (N) | 32 |

There were 6 Cip resistant isolates altogether with their sensitive controls. Table 15 summarizes QRDR alterations of amino acids in *gyrA* of those Enterobactereaceae tested Three *E. coli* isolates (4) with MIC of 128 mg/L, one from Malaysia and two from Thailand, showed a point mutation at Ser-83 causing a substitution by Leu (Ser-83-Leu) and a mutation at Asp-87 causing a substitution by Tyr (Asp-87-Tyr). The fourth *E. coli* isolate (3) from Malaysia also had a Leu substitution at 83 (Ser-83-Leu) but an Asn substitution at 87 (Asp-87-Asn), this caused the lower MIC of 32

mg/L. The fifth isolate was *C. freundii* (6) with MIC of 128 mg/L, from Thailand. This isolate had an Ile substitution at position 83 for Thr (Thr-83-Iso); we have determined that Thr is normal at this position in sensitive isolates of this species (5). This isolate had a mutation Asp-87-Tyr, similar to those mutations found in the highly-resistant *E. coli* (MIC of 128 mg/L). Another resistant isolate was an *Ent. sakazakii* T77 (8) with MIC of 32 mg/L, also from Thailand. This isolate had Tyr at position 83 and Asn at 87 while its sensitive control isolate (7), like in *E. coli*, had Ser at 83 and Asp at 87. Interestingly, a mutation to 87-Asn gave lower resistance than mutation to Tyr in both *E. coli* and *Ent. sakazakii*. None of the above mutations was found in 6 quinolone sensitive clinical *E. coli* isolates from Malaysia. Some nucleotide mutations were found outside QRDR in some resistant isolates but producing no amino-acid mutation.

There were 45 nucleotides of sensitive *C. freundii* CitS (5) which were found to be different from that of ECGYRA, resulting in 5 different amino acids at position 83, 112, 127, 128 and 155 (2 changes within QRDR: Ser-83-Thr and Ile-112-Val; 3 changes outside QRDR: Leu-127-Met, Ala-128-Ser and Ile-155-Lys).

There were 45 nucleotides of sensitive *Ent. sakazakii* isolate NCTC5920 (7) which were different to that of sensitive *E. coli* ECGYRA, giving no amino acid change. These were no other *gyrA* amino acid residues found to be associated with Cip resistance other than those at 83 and 87 in all these Malaysia and Thailand's clinical isolates.

There were some silent alterations in nucleotide sequence at position 438, 456, 483 and 516, which differed from all *E. coli* published sequences in GENBANK, and these were common to both sensitive and resistant bacteria isolated from Thailand and Malaysia (Table 16).

**Table 16 Unique nucleotides among ciprofloxacin sensitive and resistant *E. coli* isolates from Malaysia and Thailand compared to published sequences of the same species in GENBANK.**

| ***Escherichia coli*** | **BASE POSITION** | | | |
|---|---|---|---|---|
| | 438 | 456 | 483 | 516 |
| The GENBANK sequences (ECGYRA, ECGYRAD, ECGYRA1 & ECGYRAAM) | **C** | **C** | **T** | **T** |
| Six Cip-sensitive *E. coli* (KP 42, 62, 90, 111, 126 & 147) | **T** | **T** | **C** | **C** |
| Six Cip-sensitive *E. coli* (KP88, KP115, T281 & T295) | **T** | **T** | **C** | **C** |

### 4.6.2.2 RESULT: *GYRA* QRDR SEQUENCE OF NEISSERIACEAE

A 423-base region of partial *gyrA* gene was sequenced and amino acid residues at position 54 to 164 including *E. coli*'s equivalent QRDR (amino acid residues at 67-106) within brackets are shown below. The *E. coli* equivalent codons 83 and 87 are underlined. From 10) onwards red capitals in resistant sequences indicate nucleotides and amino acids different from that of *N. gonorrhoeae* NGU08817 or its control and sensitive sequence and only section where nucleotide changes occurred is shown.

9) Standard Cip sensitive-*gyrA* gene of *Neisseria gonorrhoeae* NGU08817, as listed in GENBANK (U08817), with MIC of ≤0.001 mg/L (Belland *et. al.*, 1994).

```
184 gag ctg aaa aat aac tgg aat gcc gcc tac aaa aaa tcg[gcg cgc
     E   L   K   N   N   W   N   A   A   Y   K   K   S   A   R
    54                                                    67
229 atc gtc ggc gac gtc atc ggt aaa tac cac ccc cac ggc gat tcc
     I   V   G   D   V   I   G   K   Y   H   P   H   G   D   S
                                                           83
274 gca gtt tac gac acc atc gtc cgt atg gcg caa aat ttc gct atg
     A   V   Y   D   T   I   V   R   M   A   Q   N   F   A   M
                87                        95        97   98
319 cgt tat gtg ctg ata gac gga cag]ggc aac ttc gga tcg gtg gac
     R   Y   V   L   I   D   G   Q   G   N   F   G   S   V   D
            101     103         106
364 ggg ctt gcc gcc gca gcc atg cgc tat acc gaa atc cgc atg gcg
     G   L   A   A   A   A   M   R   Y   T   E   I   R   M   A

409 aaa atc tca cat gaa atg ctg gca gac att gag gaa gaa acc gtt
     K   I   S   H   E   M   L   A   D   I   E   E   E   T   V

454 aat ttc ggc ccg aac tac gac ggt agc gaa cac gag ccg ctt gta
     N   F   G   P   N   Y   D   G   S   E   H   E   P   L   V

499 ctg ccg acc cgt ttc ccc 516
     L   P   T   R   F   P
                     164
```

10) Cip sensitive-*gyrA* gene of *N. gonorrhoeae* KP31267 (Edinburgh) with MIC of ≤0.001 mg/L.

- There was no any nucleotide changed to that of NGU08817 (9)

11) Cip resistant-*gyrA* gene of *N. gonorrhoeae* KP13843 (Edinburgh) with MIC of 0.004 mg/L.

- Altogether 2 nucleotides were changed resulting in 2 amino-acid mutations in QRDR: Ser-83-Phe and Asp-87-Gly.

```
229 atc gtc ggc gac gtc atc ggt aaa tac cac ccc cac ggc gat tTc
     I   V   G   D   V   I   G   K   Y   H   P   H   G   D   F
                                                           83
274 gca gtt tac gGc acc atc gtc cgt atg gcg caa aat ttc gct atg
     A   V   Y   G   T   I   V   R   M   A   Q   N   F   A   M
                 87
```

12) Cip resistant-*gyrA* gene of *N. gonorrhoeae* KP14240 (Edinburgh) with MIC of 0.008 mg/L.

- Altogether 2 nucleotides were changed resulting in 2 amino acid mutations in QRDR: Ser-83-Phe and Asp-87-Asn.

```
229 atc gtc ggc gac gtc atc ggt aaa tac cac ccc cac ggc gat tTc
     I   V   G   D   V   I   G   K   Y   H   P   H   G   D   F
                                                           83
274 gca gtt tac Aac acc atc gtc cgt atg gcg caa aat ttc gct atg
     A   V   Y   N   T   I   V   R   M   A   Q   N   F   A   M
                 87
```

13) Cip sensitive-*gyrA* gene of *M. catarrhalis* KP 6/S (Edinburgh) with MIC of ≤0.03 mg/L.

- Twenty six nucleotides within QRDR were changed resulting in 6 amino acids different: Ile-69-Val, Ser-83-Ile, Asn-95-Pro, Ala-97-Ser, Met-98-Leu, Val-101-Met and Ile-103-Val.
- Fifty eight nucleotides outside QRDR were changed; 22 amino-acid different.

```
                197 aac tgg aaC AAG Ccc tac aaG aaa tcC[gcC cgT
                     N   W   N   K   P   Y   K   K   S   A   R
                    58                                   67
229 GtG gtc ggc gac gtG atc ggt aaG tac cac ccG cac ggc gaC ATc
     V   V   G   D   V   I   G   K   Y   H   P   H   G   D   I
    69                                                       83
274 gcG gtC tac gac acc atc gtG cgC atg gcg caG CCG ttc TcG Ctg
     A   V   Y   D   T   I   V   R   M   A   Q   P   F   S   L
                87                           95       97  98
319 cgC taC Atg ctg Gta gac ggC cag]ggc aac ttc ggT tcg gtg gac
     R   Y   M   L   V   D   G   Q   G   N   F   G   S   V   D
            101     103         106
364 ggC GAC AAc gcc gca gcc atg cgA taC acc gaa GtG cgc atg gcC
     G   D   N   A   A   A   M   R   Y   T   E   V   R   M   A
        115
409 aaG CtG GcC cat gaa Ctg ctg gcG gac CtG gaA AaG gaa acc gGt
     K   L   A   H   E   L   L   A   D   L   E   K   E   T   G

454 CGA CtG ggT GcC aac tac gaT ggC aCc gaG caG ATC ccg GCG gtC
     R   L   G   A   N   Y   D   G   T   E   Q   I   P   A   V

499 Atg ccg acc AAG AtT ccc 516
     M   P   T   K   I   P
                    164
```

14) Cip resistant-*gyrA* gene *M. catarrhalis* KP 4/R (Edinburgh) with MIC of 4.0 mg/L.

- The nucleotide sequence was exactly the same as that of the sensitive *M. catarrhalis* KP6/S (13).

**Table 17** **Conclusion of the association of QRDR alterations in *gyrA* of *N. gonorrhoeae* and *M. catarrhalis* with Cip MICs are shown below. The amino acid number is that of *gyrA* equivalence of *E. coli*.**

| Name and Country | | Amino Acid Number | | MIC of Cip (mg/L) |
|---|---|---|---|---|
| | | 83 | 87 | |
| 9) Sensitive *N. gonorrhoeae* NGU08817, | GENBANK | Ser (S) | Asp (D) | <0.001 |
| 10) Sensitive *N. gonorrhoeae* KP31267, | Edinburgh | Ser (S) | Asp (D) | <0.001 |
| 11) Resistant *N. gonorrhoeae* KP13843, | Edinburgh | Phe (F) | Gly (G) | 0.004 |
| 12) Resistant *N. gonorrhoeae* KP14240, | Edinburgh | Phe (F) | Asn (N) | 0.008 |
| 13) Sensitive *M. catarrhalis* KP 6/S, | Edinburgh | Ile (I) | Asp (D) | <0.03 |
| 14) Resistant *M. catarrhalis* KP 4/R, | Edinburgh | Ile (I) | Asp (D) | 4.0 |

There were three Cip resistant isolates altogether with their sensitive controls. Fortunately the primer T1446 & T1447 (Table 1) designed on *N. gonorrhoeae* NGU08817, did work for *Moraxella catarrhalis*. This experiment focused on resistant *M. catarrhalis* KP4/R (14) on its unusual high Cip MIC of 4.0 mg/L. Interestingly, the evidence showed that its nucleotide sequence was not different at all from that of its sensitive control strain with MIC of 0.03 mg/L. Table 17 concludes QRDR alterations, only position 83 and 87 were altered, in *gyrA* of those Neisseriaceae tested. Both sensitive (13) and resistant (14) *M. catarrhalis* had Ile-83 and Asp-87. Thus it can conclude that these two amino acids are normal for *M. catarrhalis* and the difference in sequence might be found in *parC* sequence. Additionally, this is probably be the first time that QRDR of the *gyrA* sequence of *Moraxella* has been elucidated. There were 84 nucleotides of the sensitive *M.*

*catarrhalis* (13), which were different from that of sensitive control *N. gonorrhoeae* (9 or 10), resulting in 28 different amino acids (6 within QRDR).

As expected, the sensitive control *N. gonorrhoeae* KP31267 (10) had no alterations to that of *N. gonorrhoeae* NGU08817 (9). Two resistant *N. gonorrhoeae* KP13843 (11) and KP14240 (12) had 2 amino acid mutations. One mutation that was shared in common was Ser-83-Phe. The other was Asp-87-Gly for KP13843 (11) and Asp-87-Asn for KP14240 (12). The difference at 87 is more likely to be responsible for their difference in MICs.

There were no *gyrA* amino acid residues found to be associated with Cip resistance other than those at 83 and 87 in all these clinical *N. gonorrhoeae* and *M. catarrhalis*.

### 4.6.2.3 RESULT: *PARC* QRDR SEQUENCE OF ENTEROBACTERIACEAE

A 475-base region of *parC* gene was sequenced and amino acid residues at position 26 to 135 including *E. coli*'s equivalent QRDR (amino acid residues at 67-106) within brackets are shown below. The *E. coli gyrA* equivalent codons at 83 and 87 are underlined. From 16) red capitals in resistant sequence indicate nucleotides and amino acids different from that of ECOPARC or its control and sensitive sequence and only section where nucleotide changes occurred is shown.

15) Standard Cip sensitive-*parC* gene of *E. coli* ECOPARC, as listed in GENBANK (g147105), with MIC of ≤0.031 mg/L (Kato *et. al.* 1990).

```
 92 atg tac gtg atc atg gac cgt gcg ttg ccg ttt att ggt gat ggt
     M   Y   V   I   M   D   R   A   L   P   F   I   G   D   G
    26
137 ctg aaa cct gtt cag cgc cgc att gtg tat gcg atg tct gaa ctg
     L   K   P   V   Q   R   R   I   V   Y   A   M   S   E   L

182 ggc ctg aat gcc agc gcc aaa ttt aaa aaa tcg[gcc cgt acc gtc
     G   L   N   A   S   A   K   F   K   K   S   A   R   T   V
                                             67
227 ggt gac gta ctg ggt aaa tac cat ccg cac ggc gat agc gcc tgt
     G   D   V   L   G   K   Y   H   P   H   G   D   S   A   C
                                             83
272 tat gaa gcg atg gtc ctg atg gcg caa ccg ttc tct tac cgt tat
     Y   E   A   M   V   L   M   A   Q   P   F   S   Y   R   Y
        87
317 ccg ctg gtt gat ggt cag]ggg aac tgg ggc gcg ccg gac gat ccg
     P   L   V   D   G   Q   G   N   W   G   A   P   D   D   P
                        106
362 aaa tcg ttc gcg gca atg cgt tac acc gaa tcc cgg ttg tcg aaa
     K   S   F   A   A   M   R   Y   T   E   S   R   L   S   K

407 tat tcc gag ctg cta 421
     Y   S   E   L   L
                    135
```

16) Six Cip sensitive-*parC* gene of *E. coli* KP 42, 62, 90, 111, 126 and 147 (Malaysia) with MIC of ≤0.031 mg/L.

- The nucleotide sequence was exactly the same as that of the control *E. coli* ECOPARC (16).

17) Cip resistant-*parC* gene of *E. coli* isolate KP115 (Malaysia) with an MIC of 32 mg/L.

- One nucleotide within QRDR were changed resulting in 1 amino-acid mutation: Ser-83-Ile.
- One nucleotide outside QRDR were changed; no amino-acid mutation.

```
227 ggt gac gta ctg[ggt aaa tac cat ccg cac ggc gat aTc gcc tgt
     G   D   V   L   G   K   Y   H   P   H   G   D   I   A   C
                     75                              83
272 tat gaa gcg atg gtc ctg atg gcg caG ccg ttc tct tac cgt tat
     Y   E   A   M   V   L   M   A   Q   P   F   S   Y   R   Y
         87
```

18) Cip resistant-*parC* gene of *E. coli* isolate KP88 (Malaysia), T281 & T295 (Thailand); all with MICs of 128 mg/L.

- The nucleotide sequence was exactly the same as that of KP115 (17).

19) Cip sensitive-*parC* gene of *C. freundii* CitS (Edinburgh) with MIC of 0.012 mg/L.

In comparison to *E. coli* ECOPARC (15):

- 6 nucleotides within QRDR were changed resulting in no amino acids different;
- 20 nucleotides outside QRDR were changed; no amino acids different.

```
 92 atg tac gtC atc atg gac AgG gcg ttg ccA ttt att ggC gat ggt
     M   Y   V   I   M   D   R   A   L   P   F   I   G   D   G
    26
137 Ttg aaa ccC gtt cag cgT cgc att gtg tat gcA atg tcC gaa ctg
     L   K   P   V   Q   R   R   I   V   Y   A   M   S   E   L

182 ggG ctg aat gcc agc gcc aaa ttt aaa aaa tcC[gcc cgt acc gtc
     G   L   N   A   S   A   K   F   K   K   S   A   R   T   V
                                                67
227 ggt gac gtG ctg ggt aaa tac caC ccg cac ggc gaC agc gcc tgC
     G   D   V   L   G   K   Y   H   P   H   G   D   S   A   C
                                            83
272 tat gaa gcg atg gtG ctg atg gcg caG ccg ttc tct tac cgt tat
     Y   E   A   M   V   L   M   A   Q   P   F   S   Y   R   Y
        87
317 ccg ctg gtt gat ggt cag]ggA aac tgg ggG gcg ccg gac gat ccC
     P   L   V   D   G   Q   G   N   W   G   A   P   D   D   P
                        106
362 aaa tcC ttc gcg gcG atg cgt taT acc gaa tcc cgC ttg tcT aaa
     K   S   F   A   A   M   R   Y   T   E   S   R   L   S   K

407 tat tcc gag ctg cta 421
     Y   S   E   L   L
                    135
```

20) Cip resistant-*parC* gene of *C. freundii* isolate T1510 (Thailand) with MIC of 128 mg/L. In comparison to the sensitive control (19):

- 1 nucleotides within QRDR were changed resulting in no amino-acid mutation;
- 7 nucleotides outside QRDR were changed; 2 amino-acid mutations: Ser-132-Ala and Leu-134-Val.

```
272 tat gaa gcg atg gtg ctg atg gcg caA ccg ttc tct tac cgt tat
     Y   E   A   M   V   L   M   A   Q   P   F   S   Y   R   Y
         87

317 ccg ctg gtt gat ggt cag]ggG aac tgg ggg gcg ccg gac gat ccc
     P   L   V   D   G   Q   G   N   W   G   A   P   D   D   P
                         106
362 aaa tcc ttc gcg gcg atg cgt tat acc gaa tcc cgc ttg tct aaa
     K   S   F   A   A   M   R   Y   T   E   S   R   L   S   K

407 tat GcG gaA GtC ctG 421
     Y   A   E   V   L
         132     134
```

21) Cip sensitive-*parC* gene of *Enterobacter sakazakii* NCTC5920 with MIC of ≤0.06 mg/L. In comparison to *E. coli* ECOPARC (15):

- 11 nucleotides within QRDR were changed resulting in no amino acid different.
- 18 nucleotides outside QRDR were changed; no amino acid different.

```
 92 atg tac gtg atc atg gac cgt gcg ttg ccg ttt atC ggG gat ggt
     M   Y   V   I   M   D   R   A   L   P   F   I   G   D   G
    26
137 ctg aaG ccC gtt cag cgc cgc atC gtC tat gcg atg tcC gaa ctg
     L   K   P   V   Q   R   R   I   V   Y   A   M   S   E   L

182 ggG ctg aat gcT agc gcc aaG ttt aaG aaa tcC[gcc cgt acc gtc
     G   L   N   A   S   A   K   F   K   K   S   A   R   T   V
                                                67
227 ggC gac gtG ctg ggt aaa tac cat ccg cac ggc gaC agc gcc tgC
     G   D   V   L   G   K   Y   H   P   H   G   D   S   A   C
                                                83
272 tat gaa gcg atg gtG ctg atg gcC caG ccg ttc tct taT cgC tat
     Y   E   A   M   V   L   M   A   Q   P   F   S   Y   R   Y
        87
317 ccg ctg gtG gat ggC cag]ggg aac tgg ggG gcg ccg gac gat ccg
     P   L   V   D   G   Q   G   N   W   G   A   P   D   D   P
                        106
362 aaa tcC ttc gcg gca atg cgt taT acc gaa tcc cgC Ctg tcT aaa
     K   S   F   A   A   M   R   Y   T   E   S   R   L   S   K

407 tat tcc gag ctg cta 421
     Y   S   E   L   L
```

22) Cip resistant-*parC* gene of *Enterobacter sakazakii* T77 (Thailand) with MIC of 32 mg/L. In comparison to the sensitive control (21):

- one nucleotide within QRDR was changed resulting in one amino-acid mutations: Ser-83-Ile;
- two nucleotides outside QRDR were changed; one amino-acid mutation: Ser-60-Thr.

```
 92 atg tac gtg atc atg gac cgt gcg ttg ccg ttt atc ggg gat ggC
     M   Y   V   I   M   D   R   A   L   P   F   I   G   D   G

137 ctg aag ccc gtt cag cgc cgc atc gtc tat gcg atg tcc gaa ctg
     L   K   P   V   Q   R   R   I   V   Y   A   M   S   E   L

182 ggg ctg aat gct aCc gcc aag ttt aag aaa tcc[gcc cgt acc gtc
     G   L   N   A   T   A   K   F   K   K   S   A   R   T   V
                     60                          67
227 ggc gac gtg ctg ggt aaa tac cat ccg cac ggc gac aTc gcc tgc
     G   D   V   L   G   K   Y   H   P   H   G   D   I   A   C
                                                     83
```

**Table 18** **Conclusion of the association of QRDR alterations in *parC* of *E. coli*, *C. freundii* and *Ent. sakazakii* with Cip MICs are shown below. The amino acid number is that of *gyrA* equivalence of *E. coli*.**

| Name and Country | Amino Acid Number | | MIC (mg/L) of Cip |
|---|---|---|---|
| | 83 | 87 | |
| 15) Sensitive *E. coli* ECOPARC, GENBANK | Ser (S) | Gln (E) | ≤0.031 |
| 16) Sensitive *E. coli* (6 isolates), Malaysia | Ser (S) | Gln (E) | ≤0.031 |
| 17) Resistant *E. coli* KP115, Malaysia | Ile (I) | Gln (E) | 32 |
| 18) Resistant *E. coli* KP88, T281 & T295, Malaysia | Ile (I) | Gln (E) | 128 |
| 19) Sensitive *C. freundii* CitS Edinburgh | Ser (S) | Gln (E) | 0.012 |
| 20) Resistant *C. freundii* T1510, Thailand | Ser (S) | Gln (E) | 128 |
| 21) Sensitive *Ent. sakazakii* NCTC5920, NCTC | Ser (S) | Gln (E) | <0.06 |
| 22) Resistant *Ent. sakazakii* T77, Thailand | Ile (I) | Gln (E) | 32 |

Fortunately, the primers V4640 and V4641 (Table 1) designed on *E. coli*'s *gyrA* sequence amplified *C. freundii* and *Ent. sakazakii*. Table 18 summarizes QRDR alterations in *parC* of these bacteria. *ParC* sequences revealed here in all nine Malaysia and Thailand *E. coli* isolates displayed that there were no unique nucleotide alterations as had been found in the *E.* coli's *gyrA* sequence (2, 3 & 4). In fact, there were only two nucleotide changes and only detected in 3 resistant *E. coli* isolates (16, 17 & 18): one was a-298-g and silent, the other was g-264-t causing Ser-83-Ile. This amino acid mutation was common among the three resistant isolates regardless of whether the MIC was 32 or 128 mg/L. It is likely, therefore, that the *gyrA* gene is more responsible for Cip resistance than *parC* gene in *E. coli*.

A 330-bp sequence of *C. freundii*'s and *Ent. sakazakii*'s *parC*, including their QRDR, were revealed for the first time. No difference in amino acids was discovered between the GENBANK *E. coli* sequence and that of *C. freundii* (19); but there were 26 nucleotides different and 6 were within QRDR. Between the sensitive and resistant *C. freundii* isolates (20), there were no amino acid changes within QRDR but two mutations outside QRDR. Neither mutations is likely to have an effect on the MICs, nor is the Ser-60-Thr of the resistant *Ent. sakazakii* isolate (22) which is outside the QRDR. The resistant isolate, however, had a mutation within QRDR: Ser-83-Ile. Again, there was no difference in amino acid between *parC* genes of *Ent. sakazakii* and the GENBANK *E. coli*; nevertheless, there were 29 different nucleotides (11 within QRDR) were found between sensitive *Ent. sakazakii* NCTC5920 (21) and the GENBANK *E. coli* ECOPARC (15).

## 4.6.2.4 RESULT: *PARC* QRDR SEQUENCE OF NEISSERIACEAE

A 496-base region of partial *parC* gene was sequenced and amino acid residues at position 22 to 147 including *E. coli's* equivalent QRDR (amino acid residues at 75-115) within brackets are shown below. The *E. coli gyrA* equivalent codons 83 and 87 are underlined. From 16) the red capitals in the resistant sequence indicate nucleotides and amino acids different from that of *N. gonorrhoeae* NGU08817 or its control. Only the section where nucleotide changes occurred is shown.

23) Standard Cip sensitive-*parC* gene of *N. gonorrhoeae* NGU08907, as listed in GENBANK (g529409), with MIC of ≤0.001 mg/L (Belland *et. al.*, 1994).

```
 76 ctc gaa tac gcc atg agc gtg gtc aaa ggc cgc gcg ctg cct gaa
     L   E   Y   A   M   S   V   V   K   G   R   A   L   P   E
    22
121 gtt tca gac ggc caa aag ccc gtg cag cgg cgc att ttg ttt gcc
     V   S   D   G   Q   K   P   V   Q   R   R   I   L   F   A

166 atg cgc gat atg ggt ttg acg gcg ggg gcg aag ccg gtg aaa tcg
     M   R   D   M   G   L   T   A   G   A   K   P   V   K   S

211[gcg cgc gtg gtc ggc gag att ttg ggt aaa tac cat ccg cac ggc
     A   R   V   V   G   E   I   L   G   K   Y   H   P   H   G
    67
256 gac agt tcc gcc tat gag gcg atg gtg cgc atg gct cag gat ttt
     D   S   S   A   Y   E   A   M   V   R   M   A   Q   D   F
        83              87
301 acc ttg cgc tat ccc tta atc gac ggc atc]ggc aac ttc ggt tcg
     T   L   R   Y   P   L   I   D   G   I   G   N   F   G   S
                                       106
346 cgc gac ggc gac ggg gcg gcg gcg atg cgt tac acc gaa gcg cgg
     R   D   G   D   G   A   A   A   M   R   Y   T   E   A   R

391 ctc acg ccg att gcg gaa ttg ctg ttg tcc gaa atc aat cag ggg
     L   T   P   I   A   E   L   L   L   S   E   I   N   Q   G

436 acg gtg gat ttt atg ccg
     T   V   D   F   M   P
                    147
```

24) Cip sensitive-*gyrA* gene of *N. gonorrhoeae* KP31267 (Edinburgh) with MIC of ≤0.001 mg/L.

- One nucleotide outside QRDR was changed resulting in no amino-acid mutation.

```
391 ctG acg ccg att gcg gaa ttg ctg ttg tcc gaa atc aat cag ggg
     L   T   P   I   A   E   L   L   L   S   E   I   N   Q   G
```

25) Cip resistant-*parC* gene of *N. gonorrhoeae* KP13843 (Edinburgh) with MIC of 0.004 mg/L.

- Two nucleotides within QRDR were changed resulting in one amino-acid mutations: Glu-87-Gly.
- One nucleotide outside QRDR were changed; no amino-acid mutation.

```
256 gac agt tcc gcc tat gGg gcg atg gtg cgc atg gct cag gat ttt
     D   S   S   A   Y   G   A   M   V   R   M   A   Q   D   F
        83                87
301 acc ttg cgc taC ccc tta atc gac ggc atc]ggc aac ttc ggt tcg
     T   L   R   Y   P   L   I   D   G   I   G   N   F   G   S
                                        106
346 cgc gac ggc gac ggg gcg gcg gcg atg cgt tac acc gaa gcg cgg
     R   D   G   D   G   A   A   A   M   R   Y   T   E   A   R

391 ctG acg ccg att gcg gaa ttg ctg ttg tcc gaa atc aat cag ggg
     L   T   P   I   A   E   L   L   L   S   E   I   N   Q   G
```

26) Cip resistant-*parC* gene of *N. gonorrhoeae* KP14240 (Edinburgh) with MIC of 0.008 mg/L.

- The sequence was exactly the same as that of *N. gonorrhoeae* KP14240 (24).

**Table 19 Conclusion of the association of QRDR alterations in *parC* of *N. gonorrhoeae* and *M. catarrhalis* with Cip MICs are shown below. The amino acid number is that of *gyrA* equivalence of *E. coli*.**

| Name and Country | Amino Acid Number | | MIC (mg/L) of Cip |
|---|---|---|---|
| | 83 | 87 | |
| 23) Sensitive *N. gonorrhoeae* NGU08817, | Ser (S) | Gln (E) | ≤0.001 |
| 24) Sensitive *N. gonorrhoeae* KP31267, | Ser (S) | Gln (E) | ≤0.001 |
| 25) Resistant *N. gonorrhoeae* KP13843, | Ser (S) | Gly (G) | 0.004 |
| 26) Resistant *N. gonorrhoeae* KP14240, | Ser (S) | Gly (G) | 0.008 |

Unfortunately, the primers V4642 and V4643 (Table 1) designed on *N. gonorrhoeae*'s *parC* sequence did not work for *M. catarrhalis* as stated before in 4.3.2. It is therefore not possible to conclude why *M. catarrhalis* (28) had a high Cip MIC. Table 19 summarizes the QRDR alterations in *parC* of those in neisseriaceae. The resistant *N. gonorrhoeae* KP13843 (25) had an MIC of 0.004 mg/L and KP14240 (26) had an MIC of 0.008 mg/L. The latter had exactly the same *parC* sequence and only one mutation in QRDR, Glu-87-Gly.

Figure 8 compares all *gyrA* and *parC* QRDR amino acids of resistant clinical isolates and their type strains studied here with their Cip MICs.

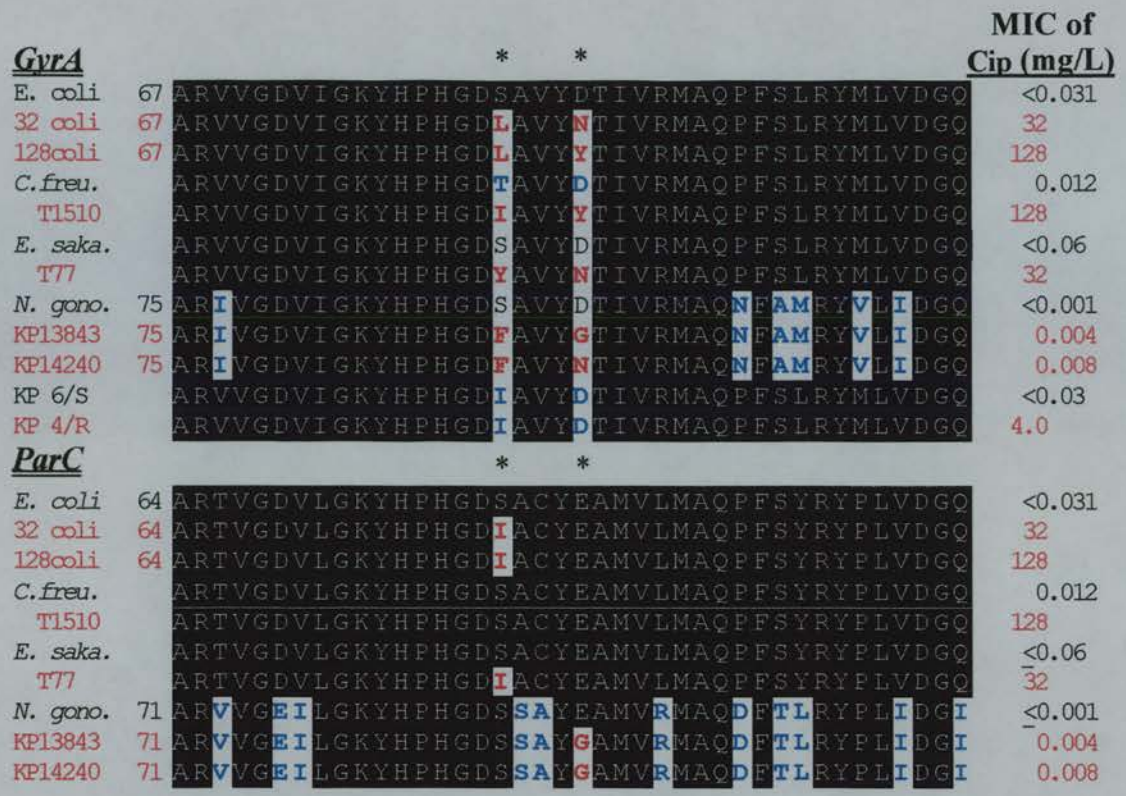


**Figure 8** **Alignment of amino acid sequences of QRDRs of the *gyrA* and *parC* from clinical bacteria. The numbers at the beginning of the sequences indicate the first amino acid of the QRDR. Letters in black indicate sequence identity, otherwise in blue or red. Letters in red indicate mutation to its sensitive control. Residues at 83 and 87 are marked by an asterisk.**

*E. coli* = Sensitive *Escherichia coli* KP42, 62, 90, 111, 126 and 147
32 coli = Resistant *Escherichia coli* KP115
128coli = Resistant *Escherichia coli* KP88, T281 & T295
*C. freu.* = Sensitive *Citrobacter freundii* CitS
T1510 = Resistant *Citrobacter freundii* T1510
*E. saka.* = Sensitive *Enterobacter sakazakii* NCTC5920
T77 = Resistant *Enterobacter sakazakii* T77
*N. gono.* = Sensitive *Neisseria gonorrhoeae* KP31267
KP13843 = Resistant *Neisseria gonorrhoeae* KP13843
KP14240 = Resistant *Neisseria gonorrhoeae* KP14240
KP 6/S = Resistant *Moraxella catarrhalis* KP 6/S
KP 4/R = Sensitive *Moraxella catarrhalis* KP 4/R

## 4.3 CONCLUSION AND DISCUSSION

Partial sequences of the *gyrA* and *parC* genes of seven resistant clinical isolates and their sensitive controls, were determined at least twice separately to assure reproducible data. All amino acid mutations (shown in red in Figure 8) found in QRDR of either *gyrA* or *parC* genes in all the isolates were at position 83 and 87 only (*E. coli*'s *gyrA* homologous position). One of the Edinburgh isolates was *M. catarrhalis* with an MIC of 4.0 mg/L. No difference was found in the *gyrA* sequence between the sensitive (KP6/S) and resistant (KP4/R) strain of *M. catarrhalis*. However, no *parC* sequences of both isolates could be achieved. Consequently, the resistant mechanism of the *M. catarrhalis* remains unresolved.

As for the six remaining isolates, they were extremely highly resistant to Cip and were from Malaysia and Thailand. Four of the six were *E. coli* and the rest were *C. freundii* and *Ent. sakazakii*. Five had three point mutations: two in *gyrA* (at 83 and 87) and one in *parC* (at 83) genes. However, there was no *parC* mutation in *C. freundii*. It is reasonable to conclude that the high resistance in these three bacterial species was largely a result of the two mutations at *gyrA* 83 and 87. Further mutations at *parC* 83 would produce even higher resistance. Four point mutations at *gyrA* 83, 87, *parC* 83 and 87 could bring the highest level of fluoroquinolone resistance as found by Vila *et. al.* (1996), who reported four mutations at 83 and 87 of *gyrA* and *parC* in a clinical *E. coli* found in Spain with the highest ever reported Cip MIC of 128 mg/L.

As only mutations at *gyrA* 83 and 87 were sufficient to generate extremely high resistance in *C. freundii* T1510 and no other mutations found in QRDR of *parC*, it reasonably leads to the suggestion that DNA gyrase is the primary target of Cip or other fluoroquinolones in *C. freundii*.

The *gyrA* Ser-83-Leu mutation is of all concern because it was found in common in all four resistant *E. coli* isolates examined here and several others with two point mutations (83 and 87 of *gyrA* or also *parC*) and very high to extremely high resistance (Cip MIC of 8-128 mg/L) (Heisig *et. al.*, 1993; Vila *et. al.*, 1996).

The mutation *gyrA* Asp-87-Tyr seems to enhance resistance more than any other mutations at this position 87 as it was found in all three resistant *E. coli* and one *C. freundii* isolates–all with Cip MICs of 128 mg/L. At the same position the less commonly-found and less resistant mutation was Asp-87-Asn encountered in two resistant isolate *E. coli* KP115 and *Ent. sakazakii* T77 both with a Cip MIC of 32 mg/L. However, KP115 and T77 had a different amino acids substitution at *gyrA*-87; Leu for KP115 and Tyr for T77. This finding is in accordance with the data by Vila *et. al.* (1996) that Asp-87-Asn was also detected in several *E. coli* isolates with Cip MIC of $\leq$64 mg/L and the *E. coli* with Asp-87-Tyr had the highest MIC of 128 mg/L.

Instead of "Ser" at *gyrA* 83, *C. freundii* have "Thr" and *M. catarrhalis* have "Ile". These Thr-83 and Ile-83 may provide both bacteria with some intrinsic resistance against fluoroquinolones according to Huang (1992), who reported Thr at position

83, instead of Ser, might be sufficient to raise the drug sensitivity by an order of magnitude as in the cases of *Klebsiella pneumoniae* and *Campylobacter jejuni*. Additionally, Huang (1994) stated that the presence of amino acids other than Ser or Thr at the 83 position appeared to render bacteria resistance to fluoroquinolone drugs at even higher levels.

It becomes clear that both amino acid positions 83 and 87, located in QRDR, of *gyrA* play significant role in extremely high resistance in bacteria studies here. The analogous *parC* 83 and 87 could also be involved in the resistant phenotype, but less significant, and *parC* 83 is likely to be more meaningful than *parC* 87. Although point mutations in all these four positions were not discovered in any one clinical isolate studied here; nevertheless, it is plausible that the homologous mutations at 83 and 87 of *gyrA* and *parC* could be a future mechanism that will produce the highest ever level of fluoroquinolone resistance in bacteria.

# 5. MUTUAL ANTAGONISM BY CIP AND RIF

It has been suggested that fluoroquinolones should be used in conjunction with a second antibacterial in order to prevent the emergence of fluoroquinolonre-resistant organisms during therapy (Scully *et. al.* 1986; Farrag *et. al.*, 1986). Clinical trials have shown that the combinations of Cip with benzylpenicillin, vancomycin (Cell wall antagonists) (Smith *et. al.*, 1988) or netilmicin (aminoglycoside) are successful in treating febrile episodes in neutropenic patients (Lewin and Smith, 1990). They could be used in combination with aminoglycosides or cell wall antagonists (Smith & Lewin, 1988.)

The use of any other drug together with fluoroquinolones does not necessarily mean that an improvement will be found in curing bacterial infections. In fact, mutual antagonism exists. Some drugs antagonise the action of quinolones and so must not be used clinically with them. For example, sub-inhibitory concentrations of chloramphenicol, rifampicin, tetracycline, clindamycin and erythromycin all antagonised the killing activity of nalidixic acid, ciprofloxacin and ofloxacin against *E. coli* KL16 (Lewin & Smith 1989). At concentration exceeding their MICs, novobiocin and coumermycin antagonised their bactericidal activities of nalidixic acid, ciprofloxacin, ofloxacin and norfloxacin against *E. coli* KL16 (Lewin & Smith 1989). In general it has been suggested that fluoroquinolones should not be combined with protein or RNA synthesis inhibitors.

In an attempt to examine mutual antagonism by chloramphenicol (Cm) and rifampicin (Rif) against Cip in *E. coli* NCTC10418, experiments were primarily set up to investigate the effects of interval exposure and removal of Cm and Rif. This should reveal the true nature of mutual antagonism.

# 5.1 BACTERIAL RESPONSE AGAINST CIP

## 5.1.1 *Escherichia coli*

As mentioned earlier in ANTIBACTERIAL ACTIVITY (page 56) all fluoroquinolones gave a biphasic bacterial survival curve when a given bacterium is treated over a range of concentrations during a single time interval. This experiment aimed to investigate whether some *E. coli* other than *E. coli* NCTC10418, had a dose response curve in the usual biphasic format. It was also to confirm the existence of the curve for an *E. coli* so that the bacteria could be used for next experiments.

## METHOD

To investigate the response of bacteria to ciprofloxacin (Cip) at various concentrations, *E. coli* KL16, *E. coli* NCTC10418 and clinical *E. coli* were grown by adding one loopful of an overnight culture of each strain into a standard vial containing 10 ml of Iso-Sensitest broth which was incubated by shaking overnight at $37^0$C. Subsequently, 0.5 ml of these cultures were inoculated into 9.9 ml of prewarmed Iso-Sensitest broth and shaked at $37^0$C for 90 minutes. Then 0.1 ml of these cell suspensions were used as inoculum. After adding inoculum into 9.9 ml of Iso-Sensitest broth in vials containing various final concentrations of Cip (0.01, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64, 1.28, 2.56, 5.12 and 10.24 mg/L) and no Cip as control, the cultures were incubated for 180 min at $37^0$C. Subsequently, spread plating viable count were performed on Columbia base agar to monitor the response.

## RESULT

It is apparent that both *E. coli* NCTC10418 and the clinical *E. coli* showed biphasic survival curves with MIC at 0.64 mg/L as the single most bactericidal concentration. However, as seen in Figure 9. *E. coli* KL16 responded in different way showing a convex curve, not the usual biphasic, with maximum kill at 10.24 mg/L which was the highest concentration used here. This ensured that not all *E. coli* responded to Cip in the same pattern and *E. coli* KL16 was more resistant to Cip than those of other *E. coli* tested. This is in contrast to the results for KL16 of some other workers and so it was decided to continue with *E. coli* NCTC10418.

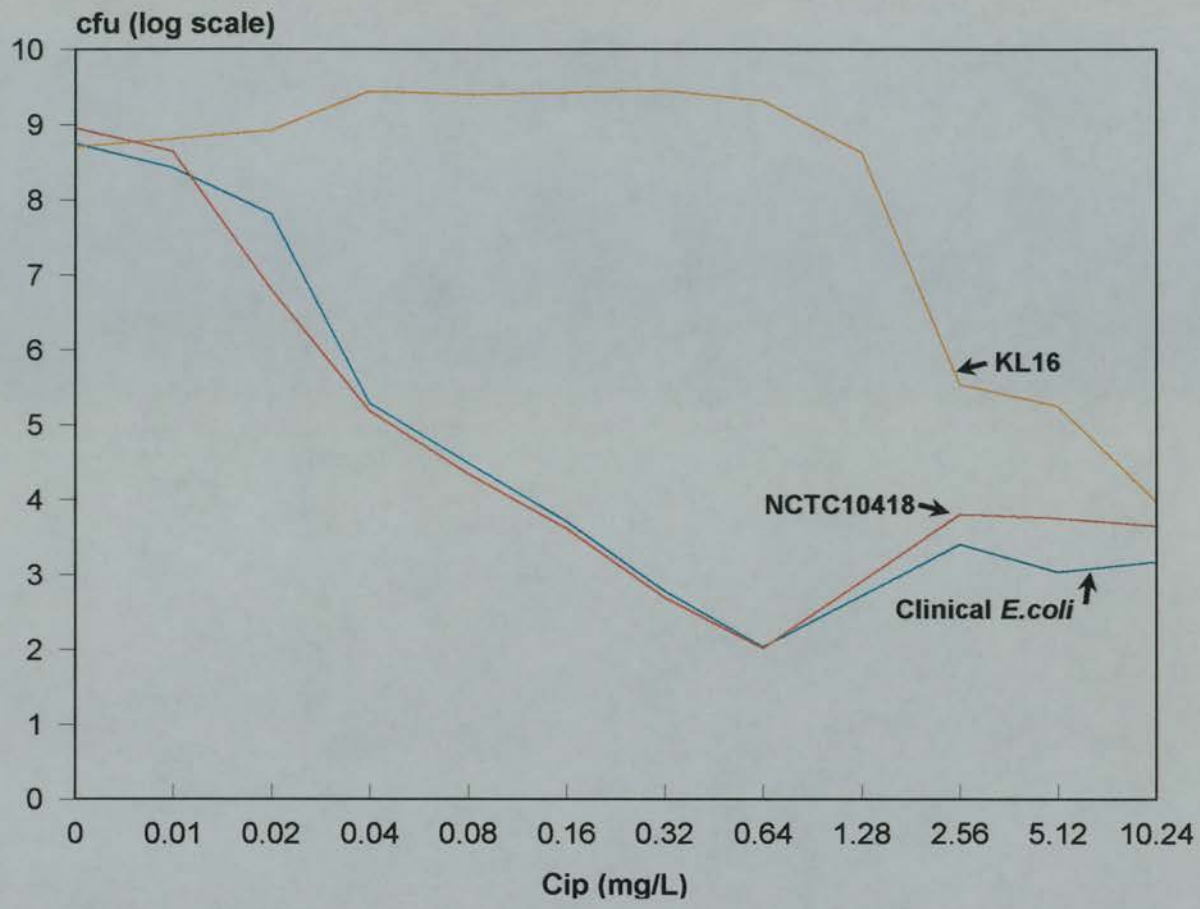


**Figure 9** **Bacterial survival curves of *E. coli* treated with Cip at concentrations 0-10.24 mg/L.**

### 5.1.2 *Staphylococcus aureus*

This experiment aimed to investigate whether a *S. aureus* exhibits the same survival curve as that of *E. coli.*

## METHOD

The same procedure applied here was the same as that used in 5.1.1 (except that the highest concentration of Cip was 2.56 mg/L) and the culture was *S. aureus* NCTC6571.

## RESULT

As seen in Figure 10 *S. aureus* did not show a biphasic survival curve and was slightly more resistant to Cip than *E. coli* NCTC10418 and the clinical *E. coli*, but much less resistant compared to *E. coli* KL16.

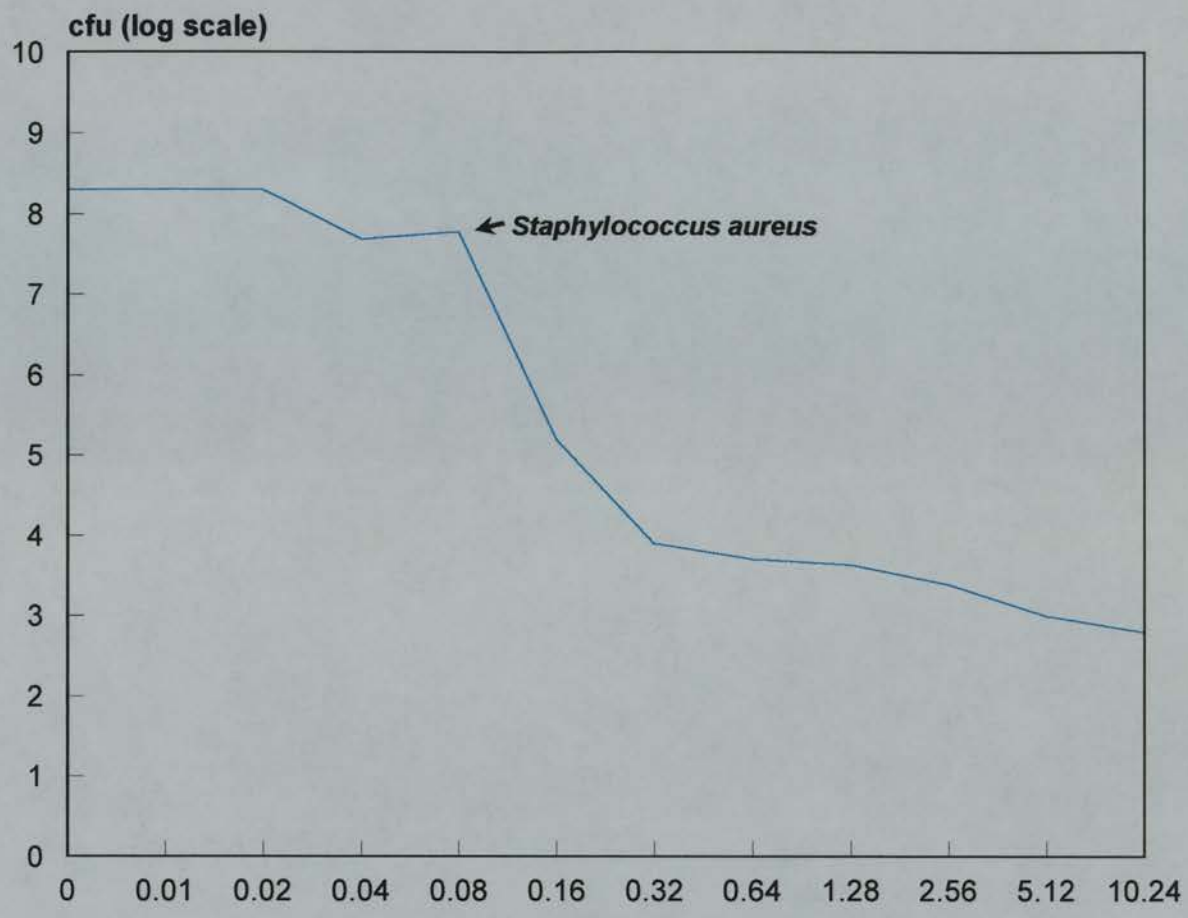


**Figure 10** **Bacterial survival curves of *Staphylococcus aureus* treated with Cip at concentrations 0-10.24 mg/L.**

## 5.2 BACTERIAL RESPONSE AGAINST Cip UNDER PROTEIN INHIBITORS

As it is commonly accepted that antibacterials such as Cm and Rif, antagonise killing activity of fluoroquinolones but there is no information available on bacterial response to the drugs when those protein inhibitors are exposed to bacteria before and after being treated with fluoroquinolones, and when Cip and Rif are removed.

### 5.2.1 INHIBITOR ADDITION AT 60 MIN AFTER Cip EXPOSURE

The purpose of this experiment was to examine the response of *E. coli* NCTC10418 towards Cip when both or each Cm and Rif were added after Cip.

### METHOD

With *E. coli* NCTC10418 a similar procedure as in 5.1.1 was carried out employing Iso-Sensitest broth containing Cip at a concentration of 0.64 mg/L. Rif and/or Cm were added to the medium to the final concentration of 20 mg/L each after 60 minute of incubation (Table 20)

**Table 20** **Pattern of antibacterial additions of Cip, Rif and Cm in experiment 5.2.1**

| Line | Incubation Time (min) | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 30 | 60 | 90 | 120 | 150 |
| 1 | Cip | ▭ | ▭ | ▭ | ▭ | ▭ |
| 2 | Cip | ▭ | Rif | ▭ | ▭ | ▭ |
| 3 | Cip | ▭ | Rif+Cm | ▭ | ▭ | ▭ |
| 4 | Cip | ▭ | Cm | ▭ | ▭ | ▭ |

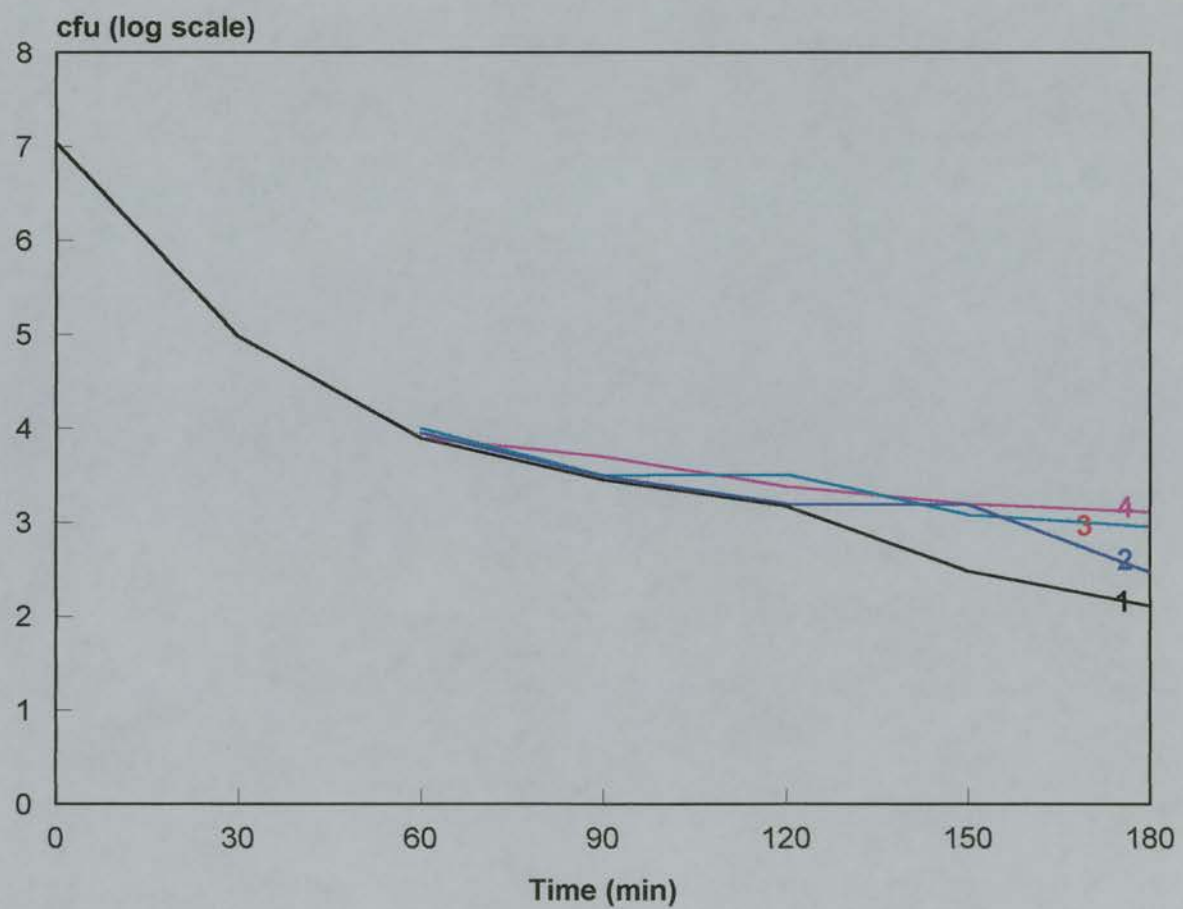


**Figure 11** **Bacterial survival curves of *Escherichia coli* NCTC10418 treated with Cip with Cm and Rif addition at 60 min after Cip exposure.**

## RESULT

Figurre 11 showed that when Rif (line 2) or Cm (line 4) or the combination of both (line 3) were in the medium, *E. coli* NCTC10418 had slightly less antagonism than with no addition (line 1). It can be concluded that the bacterial protein inhibitors antagonised the bactericidal activity of Cip. However, these differences were considerably less than they were when the drug was added at 30 mins. Perhaps the longer the bacterium was challenged, the less the antagonist effect. In any case as the difference of the effect of these three additions at 60 min on the bacteria was so small, it was considered advisable in the next experiment (5.2.2) to add the protein synthesis inhibitors earlier.

### 5.2.2 Cm ADDITION AT 0, 30 AND 60 MIN AFTER Cip EXPOSURE AND Cip ADDITION AT 0 AND 30 MIN AFTER Cm EXPOSURE

This experiment was designed to examine the antagonism of Cip with Cm by exposing *E. coli* NCTC10418 to Cm before, after and the same time as Cip treatment.

## METHOD

The procedure carried out here was the same as that in 5.2.1 except that the addition of antibacterials to the medium were changed as shown Table 21.

## RESULT

In Figure 12 there were more survivors of *E. coli* NCTC10418 when Cm and Cip were added at the beginning of the incubation – Cm addition at 0 min (line 6) compared to that with only Cip (line 3). It was, therefore, apparent that Cm antagonised Cip bactericidal activity. This antagonism seemed to be less when Cm was added at either 30 (line 4) or 60 min (line 5) after the bacteria were exposed to Cip.

**Table 21** Pattern of antibacterial additions of Cm and Cip in experiment 5.2.2

| Line | Incubation Time (min) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 | 30 | 60 | 90 | 120 | 150 | 180 |
| 1 | Cm | ▭ | ▭ | ▭ | ▭ | ▭ | ▭ |
| 2 | Cm | Cip | ▭ | ▭ | ▭ | ▭ | ▭ |
| 3 | Cip | ▭ | ▭ | ▭ | ▭ | ▭ | ▭ |
| 4 | Cip | Cm | ▭ | ▭ | ▭ | ▭ | ▭ |
| 5 | Cip | ▭ | Cm | ▭ | ▭ | ▭ | ▭ |
| 6 | Cip+Cm | ▭ | ▭ | ▭ | ▭ | ▭ | ▭ |

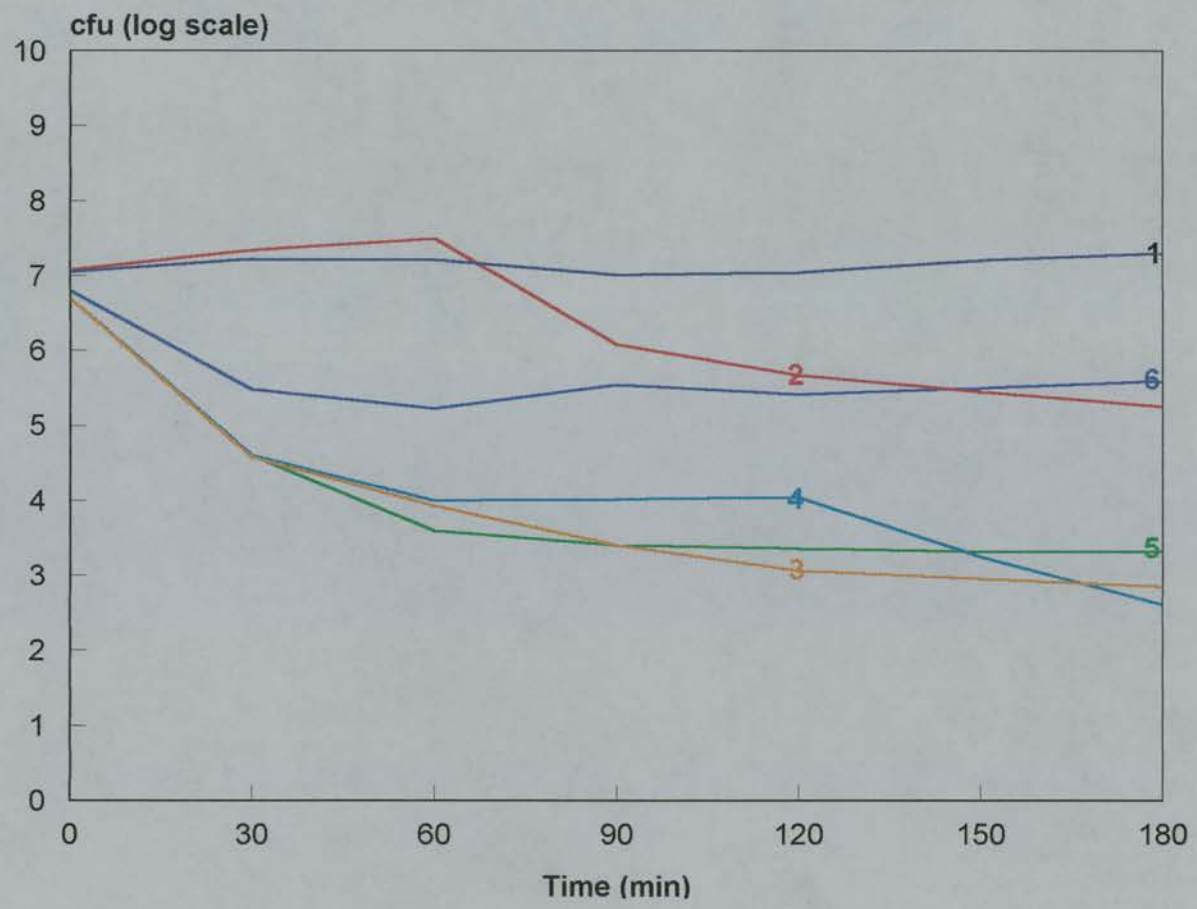


**Figure 12** Bacterial survival curves of *E. coli* NCTC10418 treated with Cm before, after and at the same time as Cip.

The antagonism appeared to be strengthened when the cells exposed to Cm 30 min prior to the addition of Cip as seen in line number 2 (Cip addition at 30 min). It was quite clear that the Cip bactericidal activity was much reduced when the organism was exposed to Cm at the same time as Cip (line 6) or when Cm was added 30 min prior to Cip (line 2) compared to the survival curve with Cip alone (line 3). In order to be certain on the antagonism caused by Cm added 30 min before (line 2), the next experiment 5.2.3 was to investigate the effect of Cm added up to 120 min before Cip.

### 5.2.3 Cip ADDITION AT 0, 30, 60, 90 AND 120 MIN AFTER Cm EXPOSURE

The aim of this experiment was to examine further the antagonism against Cip by Cm exposing *E. coli* NCTC10418 up to 120 minutes before Cip treatment.

## METHOD

The procedure carried out here was the same as that in 5.2.1 except that the presence and/or addition of Cip and Cm in the medium was as shown Table 22.

## RESULT

The result (Figure 13) showed that the earlier the Cip was added, the more cells were killed, but the killing rates seemed to be the same for each Cip addition regardless of time. This revealed that the time difference, at which the bacteria were exposed to Cm, did not affect the overall bactericidal activity of Cip. In addition Cip activity against *E. coli* NCTC10418 was decreased by Cm regardless of the time (0 to 120 min) the bacteria were previously subjected to Cm.

**Table 22** Pattern of antibacterial additions of Cm and Cip in experiment 5.2.3

| Line | Incubation Time (min) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 30 | 60 | 90 | 120 | 150 | 180 | 240 |
| 1 | Cm | ▭ | ▭ | ▭ | ▭ | ▭ | ▭ | ▭ |
| 2 | Cm | Cip | ▭ | ▭ | ▭ | ▭ | ▭ | ▭ |
| 3 | Cm | ▭ | Cip | ▭ | ▭ | ▭ | ▭ | ▭ |
| 4 | Cm | ▭ | ▭ | Cip | ▭ | ▭ | ▭ | ▭ |
| 5 | Cm | ▭ | ▭ | ▭ | Cip | ▭ | ▭ | ▭ |
| 6 | Cip+Cm | ▭ | ▭ | ▭ | ▭ | ▭ | ▭ | ▭ |

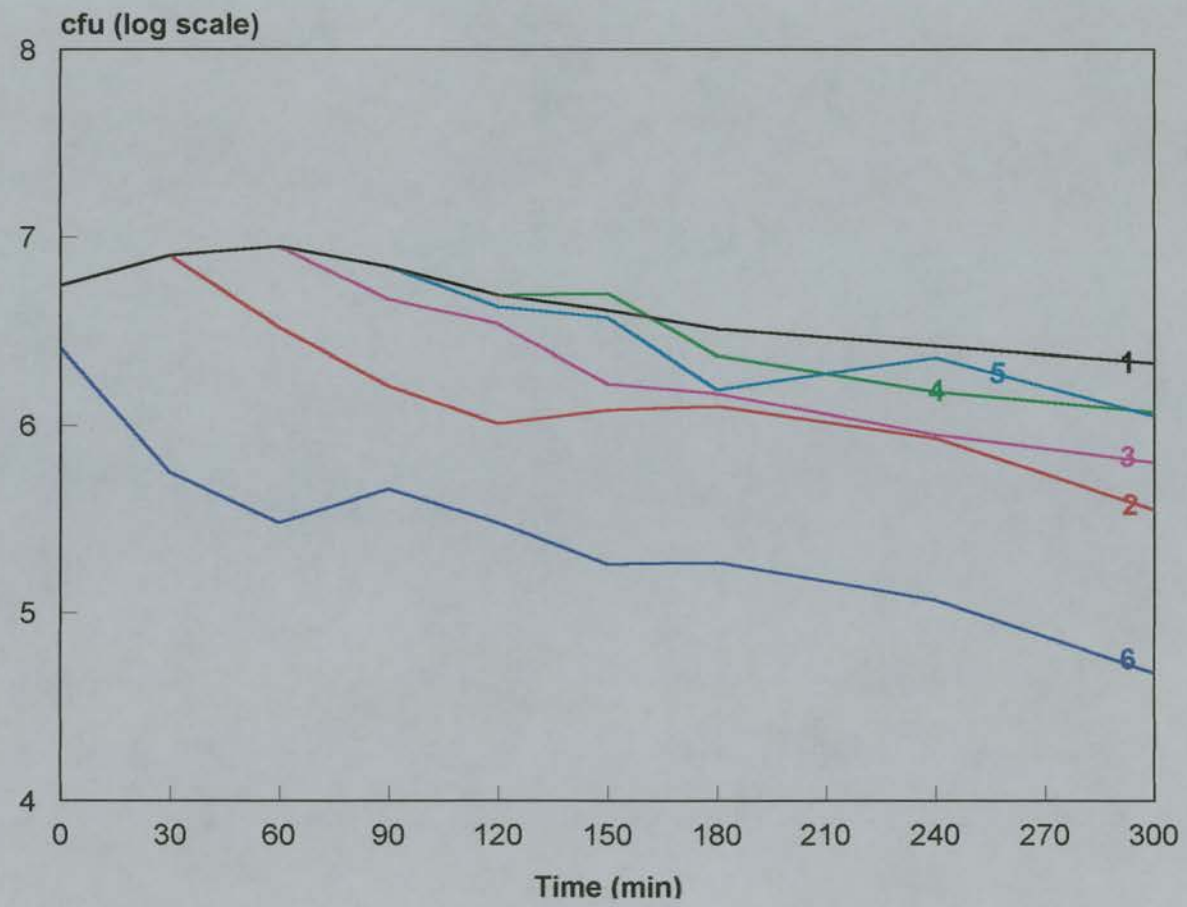


**Figure 13** Bacterial survival curves of *E. coli* NCTC10418 treated with Cm at the same time and up to 120 before Cip.

### 5.2.4 Cip ADDITION AT 0, 30, 60, 90 AND 120 MIN AFTER RIF EXPOSURE

This experiment was to investigate the effect of Rif added 0 to 120 min before Cip on bacterial response of *E. coli* NCTC10418.

## METHOD

The procedure carried out here was the same as that in 5.2.1 except that the presence and addition of Rif and Cip in the medium were as shown in the Table 23.

## RESULT

The result in Figure 14 showed apparently the same pattern as that in Figure 13 emphasizing that both bacterial protein (Cm) and RNA synthesis (Rif) inhibitors were able to slow down the activity of Cip when added to the organism at least 120 min before the fluoroquinolone. Again the Cip killing rates were nearly the same.

**Table 23** Pattern of antibacterial additions of Rif and Cip in experiment 5.2.4

| Line | Incubation Time (min) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 30 | 60 | 90 | 120 | 150 | 180 | 240 |
| 1 | Rif | ⊟ | ⊟ | ⊟ | ⊟ | ⊟ | ⊟ | ⊟ |
| 2 | Rif | Cip | ⊟ | ⊟ | ⊟ | ⊟ | ⊟ | ⊟ |
| 3 | Rif | ⊟ | Cip | ⊟ | ⊟ | ⊟ | ⊟ | ⊟ |
| 4 | Rif | ⊟ | ⊟ | Cip | ⊟ | ⊟ | ⊟ | ⊟ |
| 5 | Rif | ⊟ | ⊟ | ⊟ | Cip | ⊟ | ⊟ | ⊟ |
| 6 | Cip+Rif | ⊟ | ⊟ | ⊟ | ⊟ | ⊟ | ⊟ | ⊟ |

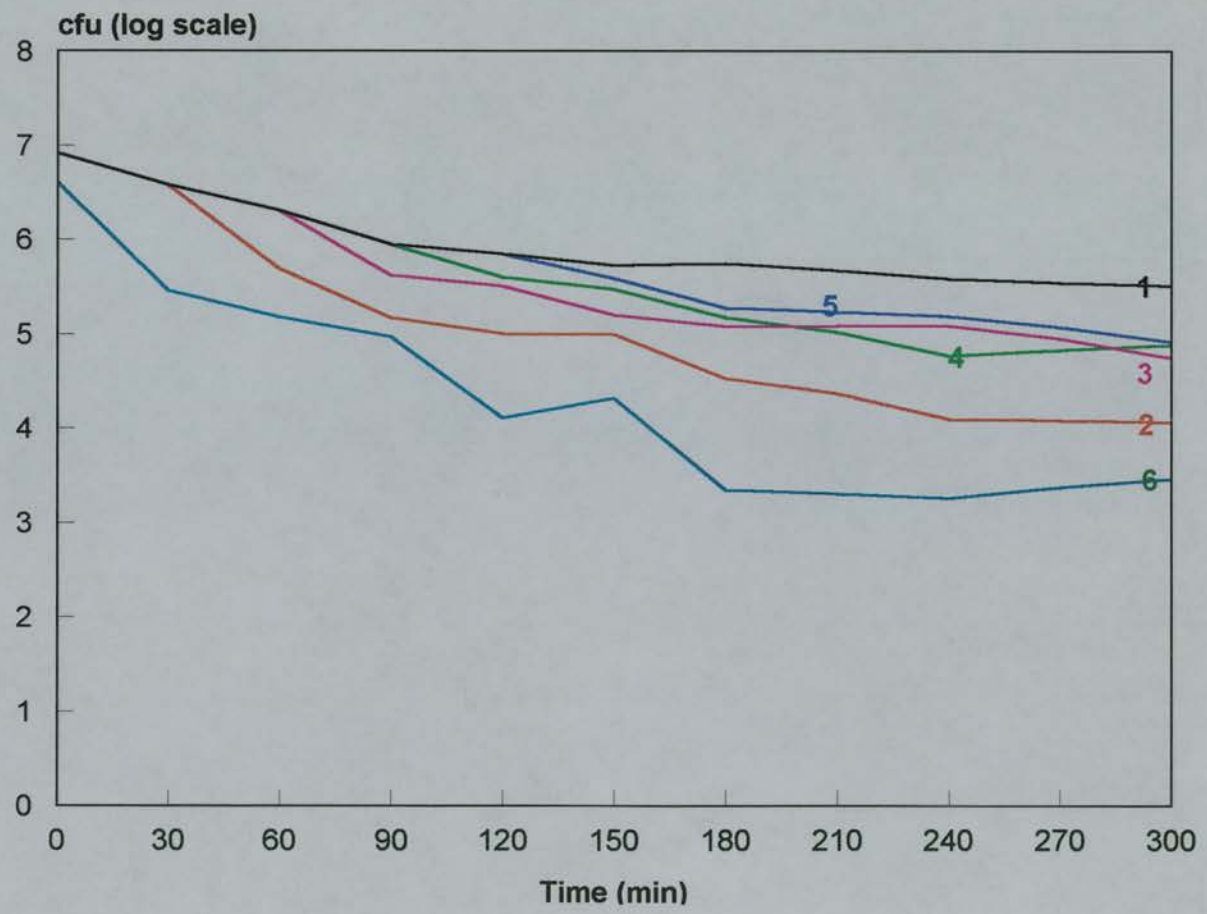


**Figure 14** Bacterial survival curves of *E. coli* NCTC10418 treated with Rif at the same time and up to 120 before Cip.

## 5.2.5 BACTERIAL PROTEIN INHIBITOR REMOVAL AFTER THE FIRST 30 MIN OF INCUBATION.

No study has reported whether or not the antagonism caused by Cm and Rif is reversible and it is, therefore, important to investigate this. This can be done by removal Cm and Rif after a fixed period of exposure. Experiments 5.2.5.1 and 5.2.5.2 were set up using the similar procedure as in 5.2.1 except for some modifications in the addition and removal of Cm, Rif and Cip; the patterns of addition and removal of Cm are shown in Table 24 and Rif in Table 25.

### 5.2.5.1 Cm removal

#### Method

After incubating *E. coli* NCTC10418 with Cm (20 mg/L) for 30 min, cells were washed to remove Cm by centrifuged at 3000g for 10 min and put into four vials containing Iso-Sensitest broth with diferent drugs. Vials contained Cm (20 mg/L), Cm (20 mg/L)+Cip (0.64 mg/L), none, or Cm (20 mg/L), respectively (Table 24). The net quantities of the medium were the same as before. The four vials were then incubated and samples withdrawn for viable counting.

## Result

Bacterial response in Figure 15 showed that Cip perform its bactericidal effect on the cells previously exposed to Cm (line 4). Nevertheless, bactericidal effect of Cip was reduced by Cm when they were put together (line 2). As expected *E. coli* NCTC10418 grew well (line 3), and remained somewhat stable when Cm had been taken out (line 1).

**Table 24** **Pattern of antibacterial additions and removals of Cm and Cip in experiment 5.2.5.1**

| Line | Incubation Time (min) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 | 30 | 60 | 90 | 120 | 150 | 180 |
| 1 | Cm | ~~Cm~~ Cm | ▭ | ▭ | ▭ | ▭ | ▭ |
| 2 | Cm | ~~Cm~~ Cm+Cip | ▭ | ▭ | ▭ | ▭ | ▭ |
| 3 | Cm | ~~Cm~~ ▭ | ▭ | ▭ | ▭ | ▭ | ▭ |
| 4 | Cm | ~~Cm~~ Cip | ▭ | ▭ | ▭ | ▭ | ▭ |

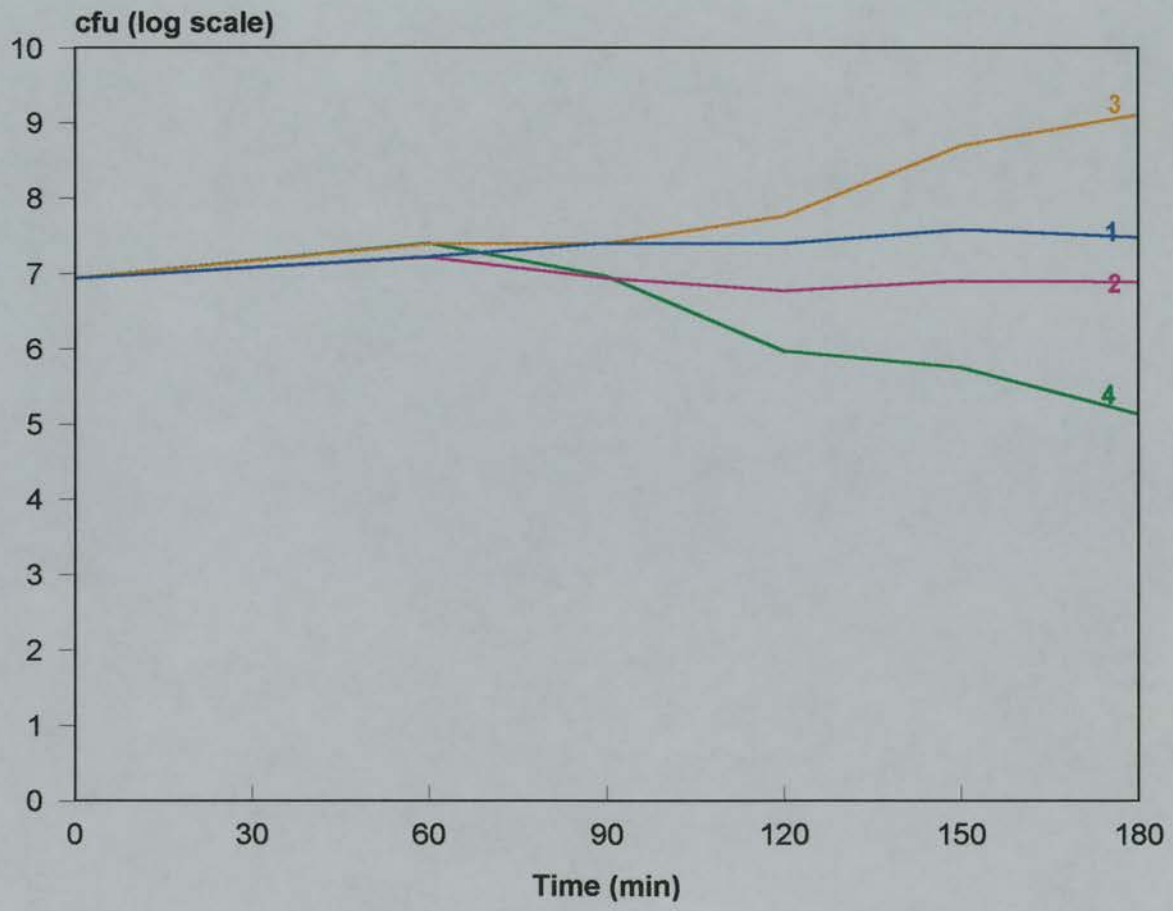


**Figure 15** **Bacterial survival curves of *E. coli* NCTC10418 when studied the effect of Cm removal on antagonism against Cip. (The pattern of addition and removal were as shown in Table 24.)**

### 5.2.5.2 Rif removal

#### Method

The procedure carried out here was the same as that in 5.2.5.1 except that Rif was used instead of Cm and the format of addition and removal of the drugs were as shown in Table 25.

#### Result

In Figure 16, it was surprising that Cip added at 30 min-incubation time together with Rif (line 2), was so active against the *E. coli* previously exposed to Rif for 30 min. The Cip killing activity against the cells (line 2) was the same as that in line 4 with Cip alone (no Rif readded). Therefore, it was concluded that Rif did not antagonise Cip activity under these conditions. As for the control treatment with no drugs (line 3) *E. coli* NCTC10418 started to grow as predicted after Rif was removed. Another control (line 1) showed that readded Rif still had a killing effect on the cells previous exposed to Rif.

**Table 25** **Pattern of antibacterial additions and removals of Rif and Cip in experiment 5.2.5.2**

| Line | Incubation Time (min) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 30 | | 60 | 90 | 120 | 150 | 180 |
| 1 | Rif | ~~Rif~~ | Rif | ▭ | ▭ | ▭ | ▭ | ▭ |
| 2 | Rif | ~~Rif~~ | Rif+Cip | ▭ | ▭ | ▭ | ▭ | ▭ |
| 3 | Rif | ~~Rif~~ | ▭ | ▭ | ▭ | ▭ | ▭ | ▭ |
| 4 | Rif | ~~Rif~~ | Cip | ▭ | ▭ | ▭ | ▭ | ▭ |

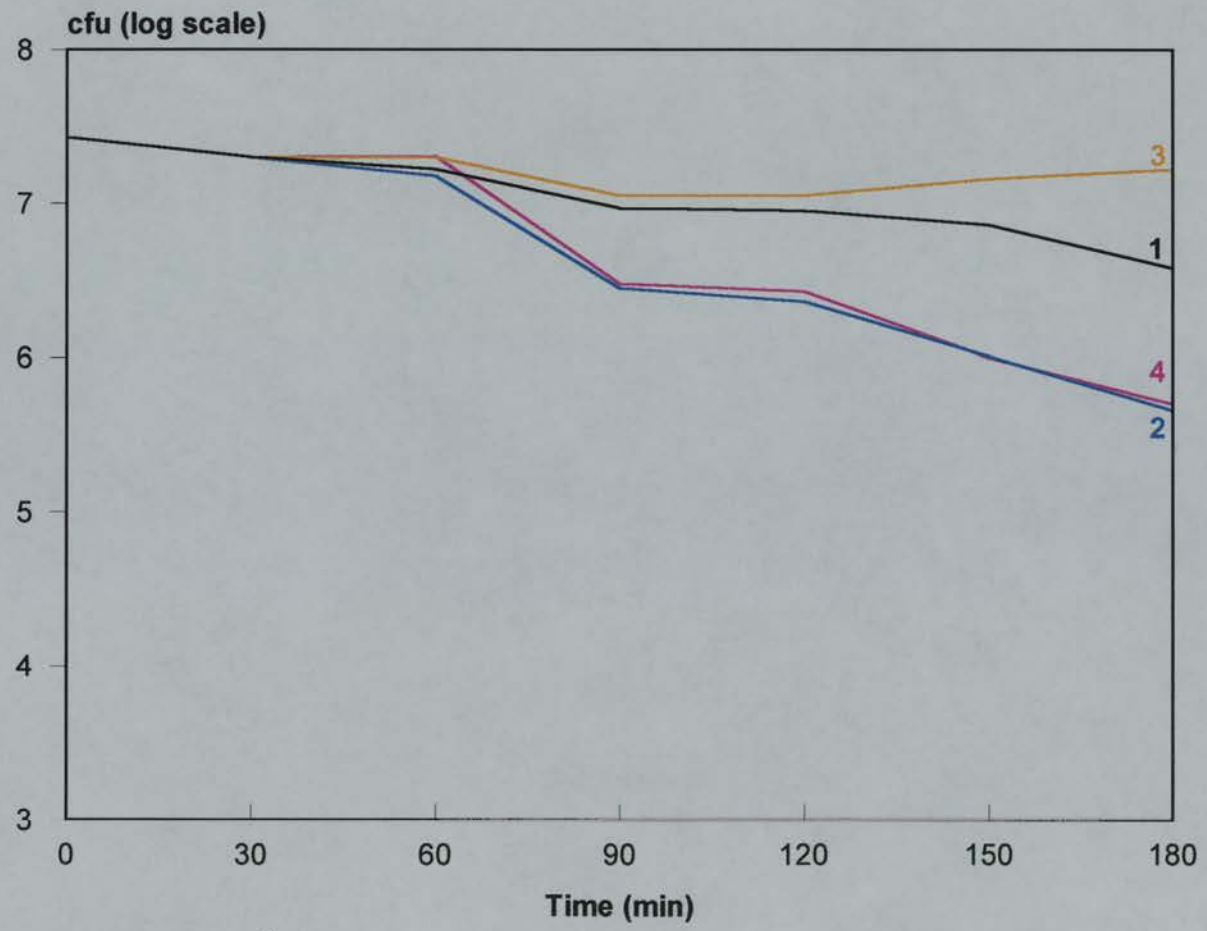


**Figure 16** **Bacterial survival curves of *E. coli* NCTC10418 when studied the effect of Rif removal on antagonism against Cip. (The pattern of addition and removal were as shown in Table 25.)**

# DISCUSSION

According to the results from experiment 5.2.3 and 5.2.4, the overall bactericidal rate of Cip was not changed by period of time (0-120 min) Cm or Rif challenged the cells. It was confirmed that Cip killing activity was antagonised by both Cm and Rif on *E. coli* NCTC10418. These results are in substantial agreement with many researchers.

It is clear that Cip regained its bactericidal effect throughout the rest of 150 mins of the experiments (5.2.5.1 & 5.2.5.2) on Cm or Rif 30 min-exposed *E. coli* when either Cm or Rif had been removed. When Cm was combined with Cip, the antagonism against Cip existed on the 30 min Cm-exposed *E. coli* as there were more survivors (line 2 in Figure 15) than that with Cip alone (line 4 in Figure 15). In marked contrast, the antagonism against by Rif vanished on the 30 min Rif-exposed *E. coli* as bactericidal activity of Cip stayed unchanged between Cip alone (line 4 in Figure 16) and Cip with Rif (line 2 in Figure 16); the same number of survivors throughout the rest of 150 mins of experiment. A possible explanation to explain why no such antagonism by Rif when *E. coli* NCTC10418 had been exposed to Rif for 30 mins, requires more information. It might be that the antagonism caused by Cm is reversible, but it is not by Rif. Nevertheless, we can no longer assume that Rif antagonises bactericidal activity of Cip on *E. coli* in all situations.

*E. coli* NCTC10418 and clinical *E. coli* did show biphasic survival curve when treated with Cip. Nevertheless, it can be concluded that not all *E. coli* responses to

Cip bactericidal activity by producing the biphasic curve; *E. coli* KL16 did show a convex curve, not the usual biphasic one. *S. aureus* NCTC6571 also did not display the biphasic but a very common curve caused by any other bactericidal drug.

# 6. DISCUSSION

## INVESTIGATION OF CIPROFLOXACIN RESISTANCE IN CLINICAL BACTERIAL ISOLATES

My findings are consistent with the premise that bacterial clinical isolates in Malaysia and Thailand were highly Cip resistant. In addition, the resistance in these urinary tract bacterial isolates was among the highest ever reported. The six *A. baumannii* found in Malaysia had very high resistance with an MIC of 64 mg/L, and one *A. baumannii* had an even higher MIC of 128 mg/L. Although clinical *E. coli* with high level resistance to Cip (MIC >1 mg/L) were rare in 1990 (Barry *et. al.*, 1990), we can no longer assume that this incidence remains low because in 1994, at least, more than 6% of such isolates in Malaysia were resistant, and this figure could be worse in Thailand. More interestingly, the level of Cip resistance of clinical *E. coli* from both Malaysia and Thailand was the highest ever reported. From Malaysia, two *E. coli* had unusually high resistance with MICs of 32 and 128 mg/L and two *E. coli* from Thailand had MICs of 128 mg/L. Vila *et. al.* (1994) previously reported the highest level of MIC against Cip for clinical *E. coli* was 128 mg/L. Some of the other screened urinary-tract isolates from Thailand which had remarkably high resistance to Cip were one *C. freundii* (128 mg/L), three *A. baumannii* (128 mg/L) and three *E.* spp. (32 mg/L). Bajaksouzian *et. al.* (1997) had previously reported that the highest level of resistance for clinical *A. baumannii* was 64 mg/L. One possible conclusion for high resistance found in Malaysia and Thailand is likely to be that the drugs have been used extensively as no strict regulation have been applied, particularly because of

the high rate of bacterial infection diseases resulting from lower standards of living, urban overcrowding, poorer sanitation and lower vaccination rates. Ill-informed attitudes towards the use of drugs, such as minor bacterial infections requiring antibiotic treatments and faliure to complete the full dose, could promote the development of drug resistance. Furthermore, if fluoroquinolones use in farming practice is proved to have some connection with human bacterial resistance, a large and prominent agriculture industry in both Thailand and Malaysia might have played a significant role in the very high prevalence of resistance found in this study. However, development of resistance to the fluoroquinolones and transfer of that resistance among animal and human pathogens has been viewed as controversial. The evidence that antibiotic use in animals was the cause of human bacterial resistance was lacking or circumstantial and has never been proved (Corpet, 1996; Tollefson, Altekruse & Potter, 1997).

Some reports suggest there is a possible connection between animal and human antibiotic resistant bacteria, for example zoonotic microorganisms can be transmitted to humans through contact with animal populations, either directly or through the consumption of contaminated food (Tollefson, Altekruse & Potter, 1997). Also, eight veterinary isolates of *E. coli* received from the Ministry of Agriculture Fisheries and Foods in the United Kingdom had fluoroquinolone resistance due to a mutation at *gyrA* Ser-83-Leu with a Cip MIC of 2 mg/L (Everett *et. al.*, 1996). Furthermore, veterinary salmonella isolates of *Salmonella* spp. had *gyrA* Ser-83-Phe and Asp-87-Gly mutations. There is also a possible link between the use of avoparcin (a glycopeptide showing cross-resistance to medically important glycopeptides), the

selection of Vancomycin-resistant enterococci (VRE) found in sewage, from stools of healthy farm animals and animal products, and and humans becoming colonized via the food chain (Bates, 1997).

With the highest MIC of Cip ever found, this study supports the report of Turnidge (1995) that developing countries showed higher antibiotic resistance as these countries often allow quinolones to be freely available over the counter for urinary tract and other simple infections. The findings of this thesis, including the Cip-resistant *Moraxella catarrhalis* (MIC of 4 mg/L) found in Edinburgh, points to the concern as to what these bacteria have in common that allows them became so highly resistant.

## GENETIC CHARACTERISATION OF MUTATION IN THE QRDR OF *GYRA* AND *PARC* GENES OF CIP RESISTANT BACTERIA OF CLINICAL ISOLATES

Mutation at 83 and 87 and number of mutations

Resistance to fluoroquinolones is mediated via mutations giving altered amino acids in the quinolone resistance determining region of DNA gyrase and topoisomerase IV. Specific amino acids within *gyrA* or *parC* QRDR correlate with sensitivity against fluoroquinolones because the two sequences code for a specific part of DNA gyrase and topoisomerase IV, respectively, which are the molecular targets of fluoroquinolone antibiotics. In addition, according to previous studies (Hallett and Maxwell, 1991; Oram & Fisher, 1991; Ruiz *et al.*, 1995; Yoshida *et al.*, 1990a) and confirmed by this study, the important positions for mutations are at 83 and 87 in both *gyrA* and *parC* genes; mutations here are associated with a significant increase in the resistance towards all quinolones. In fact, this study found no changes in positions in QRDR other than 83 and 87 in all the nine resistant isolates. Therefore, the significant difference in sensitivity towards fluoroquinolones must involve these crucial 83 and 87 positions. The factors surrounding these four *gyrA* and *parC* 83 and 87 positions that determine their effect on resistance are (1) that either DNA gyrase or topoisomerase IV is the primary target, (2) in which position is the mutation, (3) the number of mutations and (4) the types of amino acids before and after mutation at these positions.

(1) DNA gyrase or topoisomerase IV as the primary target

When both *gyrA* and *parC* QRDR of a given bacterium are known, it is clear that mutations at positions 83 and 87 in either *gyrA* or *parC* genes are more significant than the others in the manifestation of resistance against fluoroquinolone antibiotics.

The explanation is that either DNA gyrase or topoisomerase IV is a primary molecular target of fluoroquinolones. It is clear that DNA gyrase mutations at *gyrA* 83 and 87 are more influential than the mutations at *parC* 83 and 87 in conferring resistance for bacterial species whose DNA gyrase is the primary target of fluoroquinolone drugs, such as *E. coli* (Reece & Maxwell, 1991), *E. cloacae* (Deguchi *et. el.*, 1997b), *K. pneumoniae* (Deguchi *et. el.*, 1997c). Similarly, mutations at *parC* 83 and 87 are more influential for those species whose topoisomerase IV is the main target, such as *S. pneumoniae* (Pan *et. al.*, 1996; Munoz & Delacampa, 1996), *S. aureus* (Campa *et. al.*, 1997; EY, Trucksis & Hooper. 1996; Ferrero *et. al.*, 1994; Hooper, 1995). Topoisomerase IV might also be the primary target in the case of *E. faecalis* (Kanematsu *et. al.*, 1998).

(2) The mutation position

It seems that amino acid substituting for Ser at mutation *gyrA* 83 can either be Leu, Iso, Tyr, or Phe as found in this study. Which amino acid is substituted does not have an important role in the level of resistance; it is the loss of serine at position 83 that is more significant than the substituting amino acid. However, mutation at this position is a basic requirement for the bacterium to become resistant.

*GyrA* and *parC* 83 code for Ser which is considered a direct quinolone-binding site of the enzymes (Maxwell, 1992). Mutations at these positions are therefore a basic requirement for high resistance. Yonezawa *et. al.*, (1995b) suggested that the Asp-87 of *gyrA* gene interacts with quinolone drugs. That suggestion must also apply to Glu-87 of *parC* gene which is homologous to *gyrA* gene. In fact Glu and Asp are both acidic amino acids and act in a very similar manner. It is probable that the position 87 might be another binding site of the quinolone as mutations at this position alone have an effect on fluoroquinolone sensitivity more or less than that of the same bacterium with a mutation at 83 alone. When mutations occurred at both position 83 and 87, fluoroquinolone resistance is much higher than that when only one mutation is present either at position 83 or 87. In addition, it is certain that mutations at both 83 and 87 of either *gyrA* or *parC genes* (*gyrA* 83 and 87 or *parC* 83 and 87) are more significant than two mutations at both 83, or both 87 of *gyrA* or *parC*. So for maximum effect the two mutations must occur in the same gene. Based on published data, very high fluoroquinolone resistance (Cip MIC of $\geq$16 mg/L) seems to require at least two mutations which are usually at *gyrA* 83 and 87 in the case of *E. coli* whose primary target for fluoroquinolones is *gyrA*. Additionally, two mutations at *parC* 83 and 87 occurred in *E. coli* would produce a lower level of resistance (Cip MIC of <16 mg/L). An *E. cloacae* which had a very high Cip MIC of 25 mg/L had two mutations in *gyrA* at Ser-83 and Asp-87 and no mutation *parC* (Deguchi *et. al.*, 1997b). Similarly, for those whose primary target is topoisomerase IV such as the Gram-positive bacteria, the two mutations needed for high fluoroquinolone resistance must be at *parC* 83 and 87, rather than *gyrA* 83 and 87 (Ferrero *et. al.*, 1994; Hooper, 1995; Campa *et. al.*, 1997).

Other point mutations in *gyrA* or *parC* QRDR are of less influence as their effects are insignificant on sensitivity. Generally, as far as the type of substituting amino acid is concerned, mutations in QRDR other than at the position 83 and 87 do not play an important role on the level of resistance. However, there may be some particular substitutions in particular species of substituting amino acid at the positions other than at 83 and 87 that raise the level of resistance significantly. An example of this was found by Cambau *et. al.* (1992) who showed that an *E. coli* with one mutation at *gyrA* Gly-81-Asp had a Cip MIC of 16 mg/L. This mutation did appear to increase the MIC and has been found on just one occasion. However, it is possible that the high resistance reported by Cambau *et. al.* (1992) is largely due to mutations in *parC* genes because the researchers did not look at topoisomerase IV which was unknown at that time. This comment is probably true for some other previous studies and we would need more information on the *parC* sequence to gain more understanding of the resistance mechanism of bacteria through a combination of mutations in *gyrA* and *parC* genes. Consequently, only those published results with information on both gyrA and parC QRDR will be directly compared with the data reported in this thesis. They are probably the only sets that are fully valid.

(3) Numbers of mutations

The one simple conclusion about the number of mutations at positions 83 and 87 is that the more mutations, the higher the resistance against fluoroquinolones. It appears that two mutations at 83 and 87 positions in both *gyrA* and *parC* is the most efficient way for a bacterium to become highly resistant to fluoroquinolones. In this state we can probably assume that the drugs would not bind properly to the two target enzymes. In general, the more mutations, the higher level of resistance in all bacterial species, both Gram-positive and Gram-negative.

(4) Types of amino acids before and after mutations

Sensitive strains of *C. freundii* have a Thr at *gyrA* 83 instead of the Ser. *M. catarrhalis* has Ile at this position. These substitutions, Thr-83 and Ile-83, may provide both bacteria with some intrinsic resistance against fluoroquinolones. Huang (1992) reported Thr at position 83 instead of Ser might be sufficient to raise the drug sensitivity by an order of magnitude as in the cases of *Klebsiella pneumoniae* and *Campylobacter jejuni*. Additionally, Huang (1994) stated that the presence of amino acids other than Thr at the 83 position appeared to render even higher intrinsic resistance to fluoroquinolones.

The type of substituting amino acids may also play an important role in resistance. Tyrosine (Tyr) at *gyrA* Asp-87 mutation probably increases fluoroquinolone resistance more than by Asparagine (Asn) at the same position. In this study it seems that mutation *gyrA* Asp-87-Tyr was the only difference that caused three resistant isolates of *E. coli* (*gyrA* Ser-83-Leu, Asp-87-Tyr & *parC* Ser-83- Ile) to have a

higher MIC of 128 mg/L than another *E. coli* isolate (*gyrA* Ser-83-Leu, Asp-87-Asn & *parC* Ser-83- Ile) with Asn at the same position as Tyr and a lower MIC of 32 mg/L. The substitution of Tyr at position *gyrA* Asp-87 was associated with the highest MIC level in a *C. freundii* isolate (MIC of 128 mg/L) together with another one more mutation *gyrA* Thr-83-Iso. Furthermore, this study showed that the substitution of Asn at position *gyrA* Asp-87 was associated with an MIC of 32 mg/L in a resistant isolate of *Ent. sakazakii* (*gyrA* Ser-83-Tyr, Asp-87-Asn, & *parC* Ser-83-Ile). The higher resistance associated with Tyr substitution rather than Asn is in accordance with the report on *E. coli* by Vila *et. al.* (1996) who showed that the *gyrA* Asp-87-Tyr substitution occurred in several isolates, for example the substitutions (*gyrA*: Ser-83-Leu, Asp-87-Tyr & *parC*: Glu-87-Lys) produced a Cip MIC of $\leq$ 64 mg/L while *gyrA* Asp-87-Asn caused a lower Cip MIC of 8 mg/L in some other isolates (*gyrA*: Ser-83-Leu, Asp-87-Asn & *parC*: Glu-87-Lys). There are further individual examples of Tyr and Asn substituting at *gyrA* Asp-87, as well as some alternative substituting amino acids causing increased resistance at position 83 and 87. There are also examples of other different substituting amino acids at other mutation sites occurring in the same organism.

The role of the substituting amino acid in certain positions on fluoroquinolone resistance needs more investigation to correlate its effect on resistance level, especially when there are three or more different mutations and different substituting amino acids.

Relations of QRDR mutations and their sensitivity in the nine Cip resistant isolates

Among all the nine Cip-resistant isolates in this study, all amino acid mutations (shown in red in Figure 8) found in QRDR were only at position 83 and 87 of *gyrA* and *parC* genes. The number of mutations and whether they were in *gyrA* or *parC* genes can be used to categorise these resistant 9 isolates:

(1) four isolates with three mutations at positions ***gyrA* 83 and 87 and *parC* 83**:

- *E. coli* KP88, T281 and T295 with an MIC of 128 mg/L

  (*gyrA* Ser-83-Leu, Asp-87-Tyr & *parC* Ser-83- Ile),

- *E. coli* KP115 with an MIC of 32 mg/L

  (*gyrA* Ser-83-Leu, Asp-87-Asn & *parC* Ser-83- Ile) and

- *Ent. sakazakii* (T77) with an MIC of 32 mg/L

  (*gyrA* Ser-83-Tyr, Asp-87-Asn, & *parC* Ser-83-Ile)

(2) one isolate with two mutations at positions ***gyrA* 83 and 87**:

- *C. freundii* T1510 with an MIC of 128 mg/L

  (*gyrA* Thr-83-Iso & Asp-87-Tyr)

(3) one isolates with two mutations at ***gyrA* 83 and *parC* 87**:

- *N. gonorrhoeae* KP14240 with an MIC of 0.008 mg/L

  (*gyrA* Ser-83-Phe & *parC* Gln-87-Gly)

(4) one isolate with one mutations at ***gyrA* 83**

- *N. gonorrhoeae* KP13843 with an MIC of 0.004 mg/L

  (*gyrA* Ser-83-Phe)

(5) one isolate with not-fully-known numbers of mutations due to unsolved *parC* sequence

- *M. catarrhalis* KP4/R with an MIC of 4 mg/L

(no mutations at *gyrA* QRDR)

The relations between those mutations and level of resistance can be explained based on the factors. Three mutations (category 1) were found in four Enterobacteriaceae isolates: the first two were identical in *E. coli* (*gyrA* Ser-83-Leu, Asp-87-Tyr & *parC* Ser-83-Ile) which had the highest ever reported MICs of 128 mg/L, the third was a single *E. coli* isolate (*gyrA* Ser-83-Leu, Asp-87-Asn & *parC* Ser-83-Ile) with an MIC of 32 mg/L, and the last was one *Ent. sakazakii* isolates (*gyrA* Ser-83-Tyr, Asp-87-Asn & *parC* Ser-83-Ile) with an MIC of 32 mg/L. This is a result of the high numbers of mutations at the position 83 and 87 and two mutations occurred at *gyrA* 83 and 87 coding for both binding-site amino acids of their primary target DNA gyrase. In the case of *E. coli*, the reason that one isolate was less resistant (32 mg/L) could be due to the only identifiable difference which was an Asn at position *gyrA* Asp-87, and the other *E. coli* isolates in this category had Tyr, (which is less polar then Asn). Mutation Asp-87-Asn was also found in the *Ent. sakazakii* isolate which had the same lower MIC level as found in the *E. coli* isolate with the same Asp-87-Asn. Both *E. coli* and *Ent. sakazakii* would be less resistant if there were fewer mutations and mutation at *parC* 83 would be less significant than that at *gyrA* 83 and 87. This can be seen in the case of *E. coli* in Table 4. Moreover the resistance of these four isolates could be stronger if there is one more mutation at *parC* 87 as this would change all binding sites of both homologous enzymes.

Two mutations (category 2) caused a resistant *C. freundii* isolate (*gyrA* Thr-83-Iso & Asp-87-Tyr) which also had very high MICs of 128 mg/L. Once again, for the same reason as above, the two mutations are contributory to resistance as they code for both of the binding-site amino acids of fluoroquinolone molecular target. This isolate would become more resistant if there are more mutations at position *parC* 83 and 87.

Two mutations (category 3) caused a small degree of resistance in the isolate *N. gonorrhoeae* KP14240 (MIC of 0.004 mg/L). This is also true in some other studies. In the case of *E. coli* with mutation category 3 is likely to have a Cip MIC less than 32 mg/L; for example, an *E. coli* with mutation *gyrA* Ser-83-Leu & *ParC* Glu-87-Lys had a Cip MIC of 4 mg/L Vila *et. al.*, 1996). Despite the same number of mutations, mutation category 3 is less significant than those in category 2 which generates the assumption that substitution of not one but both binding sites of either DNA gyrase or topoisomerase IV is required for fluoroquinolones to block the two essential enzyme acitivities. In other words, both mutations at either *gyrA* or *parC* 83 and 87 are more critical than that at *gyrA* Ser-83 and *parC* Glu-87 found in the isolate KP14240, and than any other relevant combinations of the two mutations.

Nevertheless, there is a special case of mutation category 3 found in an *E. coli* with an MIC as high as that of mutation category 1. (See the section "4 Types of amino acids before and after mutations" for an explanation.

One mutation (category 4) produced lower resistance levels than that caused by mutation category 3. In this study *N. gonorrhoeae* KP13843 had a single mutation at *gyrA* Ser-83-Phe and a MIC of 0.004 mg/L which was less than that of the same

species *N. gonorrhoeae* KP14240 (MIC of 0.008 mg/L). The reason that mutation category 4 was less resistant than that in mutation category 3 is due to fewer numbers mutations at the position 83 and 87. However, the difference between these two categories is not much. Based on published data, it is likely that some other type of substituting amino acid in all 3 potential mutation sites in these category 3 and 4 isolates could make a considerable difference in the level of resistance between these two *N. gonorrhoeae*, making them much closer or further away or even make the resistance level caused by category 4 higher than that caused by category 3.

The sensitive (KP6/S) and resistant (KP4/R) isolates of *M. catarrhalis* had no mutations in *gyrA* QRDR as well as no difference in the whole region of *gyrA* sequence studied here and their *parC* sequence was not achieved. Consequently, the definite resistance mechanism of the *M. catarrhalis* cannot be concluded. It is, however, assumed that mutations at *parC* gene would be responsible for the 4 mg/L resistance of *M. catarrhalis* KP 4/R, and, as such, topoisomerase IV is believed to be its primary target of fluoroquinolones.

As stated earlier, the type of mutations at 83 and 87 also plays an important role and could increase, by two or three magnitudes of the Cip resistance level. While only mutations at *gyrA* 83 and 87 were sufficient to produce extremely high resistance in *C. freundii* T1510 and no other mutations found in the QRDR of *parC*, it leads to the reasonable suggestion that DNA gyrase is the primary target of Cip or other fluoroquinolones in *C. freundii*. It is probable that *Ent. sakazakii*'s DNA gyrase is the primary target of fluoroquinolones as the pattern of point mutations was the same as in *E. coli*. In the case of *M. catarrhalis*, although no *parC* gene sequence was

revealed, it is believed that its topoisomerase IV is the primary target of fluoroquinolones like *S. aureus* (Campa *et. al.*, 1997, Ferrero *et. al.*, 1994; Ferrero, Cameron & Crouzet, 1995; Hooper, 1995) as there were no mutations discovered in the *gyrA* gene.

One plausible conclusion could be that mutations in *gyrA* and/or *parC* at position 83 and 87 are the most important for all fluoroquinolone resistance. Although point mutations in all these four positions were not discovered in any one clinical isolate in this research; nevertheless, it is convincing to suppose that the homologous mutations at 83 and 87 of *gyrA* and *parC* could produce the highest ever level of fluoroquinolone resistance. Specifically, the four point mutations could be

*gyrA* Ser-83-Leu/Iso/Phe/Ile/Tyr/Trp,

*gyrA* Asp-87-Tyr/Asn/Lys/Val/Glu/Ala/Phe/Ser,

*parC* Ser-83-Ile/ Leu/Iso/Tyr/Phe/Trp and

*parC* Gln-87-Tyr/Asn/Lys/Val/Glu/Ala/Phe/Ser.

Unique *gyrA* mutations

The silent mutations in *gyrA* of ten clinical isolates of both Cip-sensitive and -resistant *E. coli* were also unique in the type of substituting base: c-438-t, c-456-t, t-483-c and t-516-c. These surprising four unique mutations could be used as a reference to demonstrate if an *E. coli* isolate is close to those of *E. coli* from Malaysia and Thailand.

Malaysia and Thailand perhaps share the same clinical bacterial reservoir

It is quite likely that clinical *E. coli* in Malaysia and Thailand were very close or they were the same isolate. This was inferred from the fact that resistant *E. coli* isolates with the same MIC of 128 mg/L: one from Malaysia and two from Thailand, shared the same complete both *gyrA* and *parC* nucleotide sequence of 1032 bp and, in addition, these three *E. coli* shared the same four one point-nucleotide silent mutations with five sensitive and one resistant *E. coli* from Malaysia; these silent mutations were not found in any GENBANK sequences and so they are unique. These findings implied that these ten clinical *E. coli* isolates were from the same source or the spread of the bacteria between these two neighbouring countries was very common. It is therefore plausible to assume that Malaysia and Thailand share the same clinical bacterial reservoir and that these are a quite distinct class of *E. coli*, different from those in western industrialised countries.

Primer Design

Although the approach of revealing unknown *gyrA* and *parC* sequences of some bacteria in this research here, by designing primer sequence on conserved regions of

*E. coli*'s and *N. gonorrhoeae*'s sequences, is not always ideal; it does work. As a result, part of both *gyrA* and *parC* of *C. freundii* and *Ent. sakazakii* and *gyrA* of *M. catarrhalis* have been revealed for the first time. These known sequences are, of course, vital points of information for accomplishment of those whole sequences of the bacteria and perhaps of other close related bacteria. Unfortunately, the approach did not work in case of *M. catarrhalis*'s *parC* gene and the reason must be that the primer sequence just simply did not match *parC* sequence of the bacteria as hoped. It is likely that conserved regions, from where the primers were designed, between *M. catarrhalis* and *N. gonorrhoeae* are different. At least this slim chance had been well aware as to the fact that percentage of nucleotides different betweeen the *gyrA* sequences (321 bp) of sensitive *M. catarrhalis* (13) and *N. gonorrhoeae* NGU08817 (9) were as much as 25%: 84 nucleotides different resulting in 28 amino acids different (6 within QRDR). The only time-saving method to determine the *M. catarrhalis*'s *parC* sequence is again to follow the same approach but improve the design of the primer sequence based on *parC* sequence of *P. aeruginosa* (PSEGAS), not on *N. gonorrhoeae*, since these bacteria share a higher degree of similarity (96%) in *gyrA* sequence than that of *N. gonorrhoeae* (74%). It might as well be a good idea to choose other conserved regions further away from QRDR.

First published sequence

This is the first report on the sequences of the *gyrA* and *parC* QRDR of *C. freundii* and *E. sakazakii*, and *gyrA* QRDR of *M. catarrhalis.* When compared the nucleotide sequences to the database of NCBI, BLAST (http://www3.ncbi.nlm.nih.gov/BLAST/) for similarity, the result are shown below:

- *gyrA* sequence (page 104?) of *C. freundii* CitS (5) was closest to *Klebsiella pneumoniae* (KPGYRA) with 91% (413/451) identity, and with 88% (400-451) identity with *E. coli* (ECGYRA).

- *parC* sequence (page 118?) of *C. freundii* CitS (19) was closest to *E. coli* (ECU28377, ECAE000384 and ECOPARC) with 92% (304/330) identity, and with 88% (290/329) identity with *Salmonella typhimurium* (STYPARCF).

- *gyrA* sequence (page 106?) of *Ent. sakazakii* NCTC5920 (7) was closest to *Klebsiella pneumoniae* (KPGYRA) with 91% (411/451) identity, and with 90% (406/451) identity with *E. coli* (ECGYRA).

- *parC* sequence (page 120?) of *Ent. sakazakii* NCTC5920 (21) was closest to *E. coli* (ECOPARC) with 91% (301/330) identity, and with 89% (293/329) identity with *Salmonella typhimurium* (STYPARCF).

- *gyrA* sequence (page 113?) of *M. catarrhalis* KP 6/S (13) was closest to *P. aeruginosa* (PSEGAS) with 96% (308/320) identity, and with 77% (246/319) identity with *E. coli* (ECGYRA). However, it shared only 74% (233/312) identity with *N. gonorrhoeae* (NGU08817) whose sequence had been used for the *M. catarrhalis* primer design.

As expected, *parC* sequence of *C. freundii* is closest to that of *E. coli* than any others [with only 65% (174/265) identity with *K. pneumoniae* (KPGYRA)]. Surprisingly, the *gyrA* sequence of *C. freundii* is closer to that of *K. pneumoniae* (KPGYRA) than that of *E. coli* (either those in Europe, the U.S.A. or th Asian strains described here) whose sequence had been the basis for the primer design. Similarly, the *parC* sequence of *Ent. sakazakii*, is closest to that of *E. coli* but its *gyrA* is closer to *Klebsiella pneumoniae* than that of *E. coli*. Again *M. catarrhalis* is not closest to that of *N. gonorrhoeae* whose sequence had been used for the primer design, but is, in fact, closest to *P. aeruginosa* (PSEGAS, L29417).

This study has shown the direction in which some clinical bacterial isolates became very highly resistant to fluoroquinolones. It is clear that point mutations at *gyrA* Ser-83, Asp-87, *parC* Ser-83 and Gln-87 are the most important mechanisms used by bacteria to overcome the fluoroquinolones. The greater the number of mutations in these four positions, the higher fluoroquinolone resistance is. The type of substituting amino acid in those mutations also play significant role in magnitude of resistance. Either mutation in "*gyrA* 83 and 87" or "*parC* 83 and 87" is more pronounced from one bacterium to another depending on whether DNA gyrase or topoisomerase IV is

their primary target of fluoroquinolones. There is a unique nucleotide sequence found in form of silent mutation, of *E. coli* from Malaysia and Thailand. It is assumed that Malaysia and Thailand had high levels of fluoroquinolone resistance and they both shared the same clinical bacterial reservoir, especially in case of *E. coli*. The *gyrA* sequence of *M. catarrhalis*'s is closer to that of *P. aeruginosa* rather than that of *N. gonorrhoeae*, and this relation may well apply to their *parC* sequence. The *gyrA* sequence of *C. freundii* and *E. sakazakii* is closer to that of *K. pneumoniae* (KPGYRA) rather than that of *E. coli*.

When the aim of the research is to find out the mutations that are responsible for the fluoroquinolone resistant mechanisms, it is recommended that not just *gyrA* genes, but also *parC* genes, are sequenced. Wide-scale resistance to quinolones is expected soon to develop further if existing fluoroquinolones are used irrationally. The main causes of the drug resistance are wide including careless use of the drugs, and that not being used to their maximum potential. There are potential solutions. In chemotherapy, it is perhaps necessary to combine one or two more appropriate drugs of different classes whenever a single agent of fluoroquinolones is needed in order to preserve, for the future, the antibacterial potency of fluoroquinolones especially against MRSA *S. aureus* and *P. aeruginosa*. To alleviate the future serious problem of antibiotic resistance, modification of antibacterial policies and specific measures to counteract the spread of such strains, inappropriate public attitudes towards the use of antibiotics in some countries like Thailand should be changed. Furthermore, much more attention should be paid to infection control.

## MUTUAL ANTAGONISM OF CIP BY CM AND RIF

The accepted perception of the bactericidal action of the fluoroquinolones has been the biphasic dose response demonstrated by Smith (1986). This study shows that ciprofloxacin had a similar biphasic dose response with *E. coli* NCTC 10418 as had been demonstrated previously with *E. coli* KL16 (Smith,1986). The results of this study demonstrated that *E. coli* KL16 did not give a biphasic dose response. The reason for this discrepancy with the previous literature is unclear but probably results from failure in maintaining strain integrity. Nevertheless, the results for *E. coli* NCTC 10418 were as expected and this strain was used throughout the result of the study.

The results with *Staphylococcus aureus* NCTC 6571 showed that ciprofloxacin was unable to produce a biphasic dose-response curve and that this Gram-positive strain, like others Gram-positive, did not follow the classic dose-response profile of ciprofloxacin challenge (Lewin & Smith, 1989; Smith & Lewin, 1988). Whether all other Gram-positive bacteria respond in the same manner is unknown.

The traditional method of examining the bactericidal response is to determine the kill-rate at the maximum bactericidal concentration (Smith, 1986). When *E. coli* NCTC 10418 was challenged at this concentration (0.64 mg/L), the action of the drug could be antagonised by the subsequent addition of Cm or Rif. This is exactly the same as that reported by Smith *et. al.* (1984a) and has been interpreted that there are two bactericidal mechanisms (A and B). Mechanism A is that part of the killing process that requires protein synthesis and is thus inhibited by Cm (protein synthesis inhibitor)

or Rif (RNA polymerase inhibitor). Mechanism B is that part which was deemed as protein synthesis independent.

Much of the previous work had concentrated on adding the inhibitors 30 minutes after ciprofloxacin. The initial studies in this thesis added the inhibitors at 60 minutes. The effects of these inhibitors appeared correspondingly less effective than those in the published literature (Smith, 1986; Smith *et. al.*, 1988). In other words, the inhibition of protein synthesis seemed less important. Therefore, was the kill at this stage due to Mechanism B?

When Cm was added earlier in the experiment it had a greater effect. The results adding Cm at 30 minutes after Cip showed marked similarities with published literature (Smith, 1986). Addition of Cm at the same time as Cip showed an even greater effect and if Cm was added 30 minutes before Cip, the effect was to reduce significantly the bactericidal effect of Cip. The earlier the Cm was added, the more prominent was the effect of Mechanism A and the less influence of Mechanism B. ?This suggests that protein synthesis occurs immediately on Cip challenge and any interference with this significantly reduces the bactericidal effect of this fluoroquinolone.

This experiment was extended so that Cip was added up to 120 minutes after Cm. The net result was that Cip was then incapable of producing a bactericidal effect. The conclusion from this must be that all killing by ciprofloxacin needs protein synthesis at some stage. This questions the role or existence of protein synthesis-free fluoroquinolone-induced death or mechanism B. When the RNA polymerase

inhibitor, Rif, is added at the same time intervals before Cip challenge, the same results are found, confirming that both transcription and translation are required to kill the cell with Cip.

The inhibition of protein synthesis is reversible. When Cm is added before Cip, then washed out of the cells and ciprofloxacin added, then the fluoroquinolone kills the bacteria. In marked contrast the antagonism by Rif vanished on the 30 min Rif-exposed *E. coli* as the bactericidal activity of Cip remained unchanged between Cip alone and Cip with Rif; there were the same number of survivors throughout the rest of 150 mins of experiment. It might be that the antagonism caused by Cm is reversible, but it is not by Rif. Nevertheless, we can no longer assume that Rif antagonises bactericidal activity of Cip in *E. coli* in all situations.

The conclusion from this section is that protein synthesis is required for all death with ciprofloxacin. It is known that SOS repair is needed (Howard, Pinney and Smith, 1993b) and this requires active protein synthesis. However, these results show that long-term protein synthesis is required not only to initiate cell death but some continued protein synthesis is required to sustain it. It is likely that there is more than one direct mechanism of action, perhaps the dual targets of the GyrA and ParC proteins may set off different death cascades. However, all these death cascades require protein synthesis, at some stage.

# APPENDIX

**catenane:** A complex of two or more circular nucleic acid molecules interlocked like links in a chain (Singleton & Sainsbury. 1988).

**catenate:** (1) (verb) To interlock circular nucleic acid molecules to form a catenae (hence *noun* catenation). (2) (adj.) Of e.g. spores: formed in chains (Singleton & Sainsbury. 1988).

**chloramphenicol:** An antibiotic produced by *Streptomyces venezuelae* and also made synthetically. It is bacteriostatic for a wide range of Gram-positive and Gram-negative bacteria; it also inhibits vegetative growth and sporulation in certain fungi. The antibiotic binds to the 50S subunit of prokaryotic and mitochondrial ribosomes. it inhibits peptidyl transferase and hence protein synthesis. Resistance to chloramphenicol is commonly due to a plasmid-specified enzyme, CAT, which catalyses an acetyl-CoA-dependent acetylation of the antibiotic at the C-3 hydroxy group to form a compound with little or no antibiotic activity. Chloramphenicol antagonizes those antibiotics (e.g. penicillins ) which inhibit only actively growing and dividing bacteria. Its clinical usage is limited by its toxicity (Singleton & Sainsbury. 1988).

**rifampicin:** A semisynthetic antibiotic of a family of highly substituted macrocyclic antibiotic (ansamycins) produced by *Nocardia mediterranei.* Rifampicins specifically inhibit eubacterial DNA-dependent RNA polymerase, binding to the β subunit and inhibiting inintiation of transcription. They do not inhibit mammalian RNA polymerase or bacterial primase (Singleton & Sainsbury. 1988).

**(DNA) topoisomerase** Any enzyme which converts one topological isomer of cccDNA to another; for example, most or all topoisomerases can alter the linking number (α) of a cccDNA molecule (thus altering its degree of SUPERCOILING), and some can form and resolve knots and CATENANES (i.e., the reaction may be inter- or intramolecular). All such topological conversions must involve transient breakage of DNA strands (Singleton & Sainsbury. 1988). There are two type of topoisomerases; *TYPE I* and *TYPE II.*

***Type I* (DNA) topoisomerases** A topoisomerase I (also called untwisting, relaxing, or nick-closing enzymnes or swivelases) that break only one DNA strand; in a ds cccDNA molecule, the unbroken strand is passed through the break before resealing, thus changing α in steps of one. An example of a type I topoisomerase is the ω (omega) protein (DNA topoisomerase I) of *Escherichia coli* (*E. coli* topoisomerase I or 'topo I'); this enzyme efficiently relaxes negatively (but not positively) supercoiled DNA, can introduce topological knots into ss cccDNA, and can convert complementary ss cccDNA circles into completely base-paired ds cccDNA. The ω protein is

necessary e.g.for chromosomal DNA replication (Singleton & Sainsbury. 1988).

***Type II* (DNA) topoisomerase** A topoisomerase II that break both strands of a ds cccDNA molecule; a (double-stranded) segment of DNA from elsewhere in the molecule is then passed through the break (without strand rotation) before re-sealing : this leads to changes in $\alpha$ in steps of two. Enzyme of both types have been found in prokaryotic and eukaryotic cells. Type II topoisomerases include gyrase, reverse gyrase, bacteriophage T4 topoisomerase (gp39,52,60), and topoisomerase IV.

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