Fluoroquinolone Resistance in *Escherichia coli*:

Interplay of Mechanisms, Dose Selection and Targets for Suppression

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A dissertation Presentation to

The Department of Pharmacological and Pharmaceutical Sciences

University of Houston

In Partial Fulfillment of

The Requirement for the Degree

Doctor of Philosophy

By

Renu Singh

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ABSTRACT

Antibiotic resistance emergence is a blooming health problem. The interaction between the pathogen, antimicrobial agent and the host presents a complex challenge. Attempts to suppress the emergence of resistance have been relatively unsuccessful. One of the reasons for this could be a gap in our understanding of how bacteria deal with these agents. Our studies were focused to understand the intricacies involved in the resistance development process under antibiotic selective pressure and to find strategies to suppress antibiotic resistance emergence.

Bacteria have a natural tendency to form spontaneous mutants. It is likely that a high inoculum may harbor pre-existing mutants, which could be selectively amplified if the dose exposure is sub-optimal. Using a high inoculum of *Escherichia coli,* we demonstrated that an optimal dose exposure of moxifloxacin could be selected to suppress resistance. Even at a low inoculum, multiple bacterial mechanisms, such as target site mutations and efflux pump overexpression can lead to resistance. The SOS response system is one of the mechanisms inducing mutations by the derepression of gene involved in error prone replication. We investigated the effect of *recA* deletion (the sensor of the SOS system) on the emergence of resistance in *E. coli*. Our results suggest that apart from MIC reduction, *recA* deletion/inhibition could be beneficial in delaying the fluoroquinolone resistance emergence in *E. coli*.

Efflux pump overexpression is another common mechanism implicated in antibiotic resistance. Although efflux pumps confer low-level resistance, we demonstrated that efflux mechanisms facilitate acquisition of target site mutations that eventually lead to high-level resistance. In the wild-type *E. coli*, efflux pump overexpression (*acrAB*) preceded the acquisition of target site mutations. Experiments conducted with efflux pump deleted (Δ*acrAB*) strain delayed the emergence of resistance suggesting that the inhibition of AcrAB efflux pump could be a robust strategy for slowing the development of resistance in clinically important Gram-negative bacteria. Our results from these studies have added to the present understanding of the antibiotic resistance development process and have highlighted the importance of efflux pumps in facilitating the development of high-level fluoroquinolone resistance in *E. coli*.

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CHAPTER 1

Introduction

Antibiotic resistance is a blooming public health problem. The economic burden of antibiotic resistance to the United States health care system is in excess of $20 billion annually (Taubes, 2008; Haddix et al., 2003). Every year ~ 2 million people acquire infections in the United States, of which about 90,000 die due to antibiotic resistance. A major cause of morbidity and mortality is the emergence of multidrug resistant bacteria. Despite the availability of many antibiotics, infectious diseases remain as the third leading cause of death in the United States (Spellberg et al., 2008). The impending danger of this alarming spread of antibiotic resistance threatens a return to the pre-antibiotic era. Thus, there is an urgent need to understand the mechanisms of resistance, to develop new targets for suppressing antibiotic resistance and to improve existing treatment strategies.

Fluoroquinolones constitute a widely prescribed class of antibiotics that are effective against a broad spectrum of pathogens. Fluoroquinolones possess many favorable features, such as excellent bioavailability, good tissue penetration and long half-life leading to once daily dosing. However, the frequent association of prior fluoroquinolone exposure and emergence of multidrug resistant phenotypes has created a conundrum regarding their usage (Lautenbach et al., 2004; Boyd et al., 2008). Suppressing

emergence of resistance against fluoroquinolones is crucial so we do not lose this important class of drug from our antibiotic armamentarium.

Presently, at least two main mechanisms of fluoroquinolone resistance are known. Several modifications to the chemical structure of this class have been made in an attempt to increase potency and decrease resistance emergence (Pestova et al., 2000; Beyer et al., 2000). However, these attempts to suppress the emergence of resistance have been relatively unsuccessful. One reason for this lack of success is the fundamental gap in knowledge regarding how resistance mechanisms interact at various levels of drug exposures. Some new quinolone derivatives are presently in the development stage, such as dual inhibitors, des-quinolones (Huband et al., 2007; Emrich et al., 2010). Several strategies could be used to revive older fluoroquinolones in the clinics and to guide the usage of new fluoroquinolones. Some of the strategies include the selection of optimal dosing and combination therapy with inhibitors that reduce the emergence of resistance. The objectives of our studies were designed to address these fundamental gaps in our knowledge.

Fluoroquinolones have a broad spectrum of activity against Gram-positive, Gram-negative and atypical pathogens. The antibacterial activity of fluoroquinolones is mediated by inhibition of DNA metabolic enzymes, DNA gyrase and topoisomerase IV, two essential enzymes that untangle DNA and regulate DNA supercoiling (Drlica, 1999;

Hooper, 2001). DNA gyrase is encoded by *gyrA* and *gyrB*, and topoisomerase IV is encoded by *parC* and *parE*. It is well known that bacteria attain resistance to fluoroquinolones by multiple mutations in discrete regions of the gyrase and topoisomerase IV gene known as the 'quinolone resistance determining region' (QRDR), at the site of fluoroquinolone binding (Khodursky et al., 1995). Overproduction of the efflux pumps also confers low-level resistance against fluoroquinolones (Neyfakh et al., 1993; Pumbwe et al., 2006; Chang et al., 2007). This increase in efflux pump expression can be transient or stable (caused by mutations in genes regulating the expression of these efflux pumps) (Kern et al., 2000; Wang et al., 2001; Jumbe et al., 2003; Louie et al., 2007). Plasmid-mediated quinolone resistance has also known to be implicated (Martínez-Martínez et al., 1998; Jacoby et al., 2003). Among those known so far are *qnr* (*qnrA*, *qnrB* and *qnrS*), *qepA*, *aac*(*6′*)-*Ib-cr* and *oqxAB* (Yamane et al., 2007; Wang et al., 2009; Zhao et al., 2010). These plasmids protect the bacteria from the lethal effect of the fluoroquinolones by different mechanisms.

Our work in this dissertation was mainly focused on *Escherichia coli*, which is a clinically important Gram-negative pathogen. It is the leading cause of urinary tract infections and intra-abdominal infections (Kahlmeter, 2003; Rossi et al., 2004). Fluoroquinolones are one of the commonly prescribed drugs against *E. coli* infections. Hence, our studies were directed towards understanding the mechanisms of fluoroquinolone resistance in *E. coli*. According to the European Antimicrobial

Resistance Surveillance study, prevalence of fluoroquinolone resistance to *E. coli* has increased up to 50% in major parts of Europe (Kronvall, 2010). Several surveillance studies also showed a temporal rise in fluoroquinolone resistance in the United States in recent years (Cattaneo et al., 2008; Hawkey et al., 2009).

Bacteria have a natural tendency to form spontaneous mutants. The fidelity of DNA polymerase in bacteria is not full proof. Hence, with every 10 billion nucleotides incorporated, one error is made. This error may lead to antibiotic resistance. Therefore, when the bacterial population is more than the inverse of mutational frequency to resistance, it is likely to consist of two sub-populations - susceptible and resistant (Drake, 1991; Blondeau et al., 2001). It has been demonstrated, while a sub-optimal dose exposure selectively amplifies the resistant sub-population, an optimal dose suppresses both populations (Tam et al., 2005). Intra-abdominal infections are one of the most common infections caused by *E. coli* (Rossi et al., 2006). In these infections, a high inoculum of bacteria exists and pre-existing mutants are more likely encountered. Our first study was focused on investigating the effect of dose selection on resistance emergence at a high inoculum of *E. coli*. We hypothesized that a higher drug exposure would be required to suppress resistance emergence, when pre-existing mutants were present (Singh et al., 2009).

One of the reasons for the increase in resistance to fluoroquinolones is they are potential inducers of the bacterial SOS response system (Cirz et al., 2005). SOS response is a global DNA damaging response system and is mediated by 40 or more SOS genes in *E. coli*, which are repressed by LexA protein in a non-triggered state (Erill et al., 2007). However, in case of DNA damage by a DNA damaging agent, there is formation of single stranded DNA, which activates RecA protein (Ogawa et al., 1990). Activated RecA promotes the autocatalytic cleavage of the LexA repressor of SOS genes. Derepression of SOS genes induces the programmed expression of many genes involved in error prone transcription, excision repair and cell division inhibition. This may eventually lead to mutant formation. Strategies to inhibit this process could help to suppress resistance and our second series of investigations were designed to study the effect of *recA* deletion on levofloxacin resistance emergence (Singh et al., 2010).

Various studies indicate that clinically relevant (high-level) fluoroquinolone resistant isolates of *E. coli* have multiple stepwise acquired mutations in the DNA gyrase and topoisomerase IV (Leavis et al. 2006; Morgan-Linnell et al., 2007; Dunham et al., 2010). These studies suggest that target site mutations constitute the main mechanism of resistance. In a study by Morgan-Linnell et al., 100% of the clinical isolates had target site mutations (2009). Approximately 30% of *E. coli* clinical isolates had efflux pump overproduction, which conferred low-level resistance with moderate increase (2- to 4-fold) in the MICs (Pumbwe et al., 2006; Chang et al., 2007). While in clinical isolates

efflux pumps do not appear to be as important as target site mutations, various *in vitro* and *in vivo* studies using efflux pump mutant strains suggest that efflux pumps play a central role in the emergence of resistance (Lomovskaya et al., 1999; Kern et al., 2000; Jumbe et al., 2003). The temporal interplay between these two mechanisms and the extent of contribution of efflux pumps is not well understood. It is not known if these two mechanisms are just two independent events or there is interplay between them to acquire high-level resistance in *E. coli*.

Considering that at least three highly specific mutations in the QRDR of target genes are required to confer high-level fluoroquinolone resistance, it is likely this process would take time. In contrast, efflux pumps are constitutively present and are likely to be the first defense system for the bacteria. We hypothesized that once the bacteria encounter an antibacterial agent, there is overexpression of efflux pumps to extrude the agent, lowering the intracellular drug concentration. This in turn helps in the acquisition of specific mutations in the QRDR of DNA gyrase or topoisomerase IV. However, since efflux of antibiotics is an energy-dependent process, it is likely not favored by bacteria for a long duration and eventually mutations in QRDR become the prime mechanism for resistance (Van et al., 2000). We proposed efflux pump overexpression and mutations at target sites are not two independent events, rather they follow a temporal sequence in their development and efflux pumps play an important role in 'facilitating' the acquisition of high-level resistance. We expected that in the absence of efflux pumps, it would be

difficult to acquire high-level fluoroquinolone resistance. Therefore, we compared the emergence of resistance in wild-type strain of *E. coli* to that of efflux pump deleted strain (Δ*acrAB*) to understand the role of the efflux pump in fluoroquinolone resistance emergence.

Our hypothesis is supported by similar observation in Gram-positive bacteria – *Streptococcus pneumoniae* using levofloxacin (Jumbe et al., 2003). In this study, when a wild-type *S. pneumoniae* was used in a mouse thigh infection model, the authors were unable to isolate levofloxacin resistant mutants. However, when they inoculated the mice with mutant strains overexpressing efflux pump, high-level resistant mutants were recovered. This suggested that efflux pump overexpression facilitated the acquisition of high-level resistance. *E. coli* has more than 40 putative efflux pumps, out of which three efflux pumps mediate quinolone resistance- AcrAB-TolC, MdfA and NorE. However, Yang et al. have demonstrated that the AcrAB-TolC efflux pump is the major efflux pump contributing to resistance development in *E. coli* (2003). Hence, our study is focused on the AcrAB efflux pump, which belongs to the Resistance/Nodulation/Cell Division Family (RND) (Nikaido et al., 2001).

Findings from these studies play a critical role in our attempts to understand the intricacies involved in the development of resistance and shed some light on potential targets to suppress emergence of resistance. Following the completion of this dissertation

research, important results in the following areas were achieved: (1) dosing and resistance selection in a high inoculum with pre-existing mutants; (2) impact of *recA* on levofloxacin exposure-related resistance development; (3) understanding of the temporal relationship between the two mechanisms of fluoroquinolone resistance; and the effect of deleting efflux pump on antibiotic resistance development in Gram-negative bacteria.

CHAPTER 2

Review of Relevant Literature

2.1 FLUOROQUINOLONES

2.1.1 Introduction

Fluoroquinolones are a widely prescribed class of synthetic, broad-spectrum agents with bactericidal activity (Walker, 1999; Oliphant et al., 2002). The first fluoroquinolone was introduced in 1986, and was derived by modification of a quinolone – nalidixic acid. As a group, the fluoroquinolones have excellent *in vitro* activity against a wide range of both Gram-positive and Gram-negative bacteria. Presently, fluoroquinolones are classified into four generations as shown in Table 1. This classification is based on their spectrum of activity. The older generations have activity against mostly Gram-negative bacteria whereas the newer generations have enhanced activity against Gram-positive bacteria as well.

Fluoroquinolones exhibit concentration-dependent bactericidal activity, which becomes more pronounced as the serum drug concentration increases to approximately 30 times the minimum inhibitory concentration (MIC) (Turnidge, 1999). This is opposed to β-

lactams, which exert time dependent killing and beyond a certain threshold the increase in concentration does not increase bacterial killing (Levison et al., 2009). This class of drugs have moderate to excellent bioavailability and good tissue penetration (Walker, 1999). Elimination half-lives for the quinolones vary from 1.5 to 16 h. Therefore, most drugs of this class are administered every 12 to 24 h. Most fluoroquinolones are excreted renally; except sparfloxacin, moxifloxacin, and trovafloxacin are excreted hepatically.

Generation	Spectrum of activity	Quinolones
First	Enterobacteriaceae	cinoxacin, nalidixic acid
Second		
Class I	Enterobacteriaceae	enoxacin, lomefloxacin, norfloxacin
Class II	Enterobacteriaceae, atypical pathogens, *Pseudomonas aeruginosa*	ciprofloxacin, ofloxacin
Third	Enterobacteriaceae, atypical pathogens, streptococci	levofloxacin, gatifloxacin, moxifloxacin, sparfloxacin
Fourth	Enterobacteriaceae, atypical pathogens, streptococci, anaerobes, methicillin-susceptible *S. aureus*, *P. aeruginosa*	trovafloxacin

Table 2.1: Classification of quinolones. Adapted from Oliphant et al., 2002.

2.1.2 Mechanism of action

Fluoroquinolones target two essential metabolic enzymes, DNA gyrase and topoisomerase IV. DNA gyrase is a tetramer composed of two subunits each of GyrA and GyrB, and similarly topoisomerase IV is composed of two subunits each of ParC and ParE. Gyrase is responsible for introducing negative supercoils into DNA and for relieving torsional stresses that accumulate ahead of transcription and replication complexes. Topoisomerase IV exerts a potent decatenating activity and is involved in the separation process of the DNA daughter chains after chromosome duplication (Zechiedrich et al., 1997; Hooper, 2001). Topoisomerase has also been shown to unknot by recognizing specific DNA juxtapositions (Deibler et al., 2001; Liu et al., 2006). DNA topoisomerase IV has also been reported to act in concert with gyrase in making an important contribution to the steady-state level of supercoiling in *E. coli* (Zechiedrich et al., 1990; Zechiedrich et al., 1997; Zechiedrich et al., 2000). The fluoroquinolones exert their lethality by trapping these two topoisomerases on DNA as drug/enzyme/DNA complexes in which double-strand DNA breaks are held together by protein (Drlica et al., 2008). Permanent gaps in the DNA strands induce synthesis of repair enzymes called exonucleases, initiating uncoordinated repair processes. This results in irreversible damage of DNA and death of the bacterium. Chromosome fragmentation and the resulting surge in reactive oxygen species have also been suggested to contribute to their lethal action (Drlica et al., 2009).

Depending on the type of bacterium, these enzymes represent either the primary or secondary target of antimicrobial action. In Gram-negative bacteria, such as *E. coli*, fluoroquinolones predominantly inhibit DNA gyrase, whereas for Gram-positive organisms like *Staphylococcus aureus*, topoisomerase IV is the principle target (Hooper, 2001). It has been suggested that the primary and secondary targets are determined by the ability of the fluoroquinolone to inhibit the two topoisomerases (Blanche et al., 1996). With *E. coli,* the fluoroquinolone concentration needed to inhibit DNA gyrase activity by 50% (IC_{50}) was lower (4- to 8-fold) as compared to that needed to inhibit topoisomerase IV activity. In *S. aureus*, topoisomerase IV was more sensitive to fluoroquinolones than DNA gyrase; IC_{50} values for topoisomerase IV being 3- to l0-fold lower than that of DNA gyrase. For both organisms, the MIC was closer to the IC_{50} of the more sensitive enzyme, suggesting the fluoroquinolone MIC is determined by activity against the primary (more susceptible) target.

2.1.3 Mechanisms of resistance

Resistance to fluoroquinolones appeared immediately after the introduction of these agents into clinical practice (Acar et al., 1997). Mechanisms of fluoroquinolone resistance can be largely classified based on chromosomally-mediated or plasmid-mediated mechanisms. In chromosomally-mediated mechanisms, resistance is acquired by alterations in the target site of fluoroquinolone binding and overexpression of

chromosomally encoded efflux pumps.

Resistance to fluoroquinolones mostly occurs because of stepwise mutations in the genes encoding DNA gyrase and topoisomerase IV. These mutations are localized to the QRDR of each subunit type, at the active site of the enzyme. The most commonly occurring mutations result in amino acid substitutions/deletions in *gyrA* and *parC* (Hane et al., 1969; Yoshida et al. 1988; Heisig, 1996). In *gyrA*, these alterations are mostly at codon 83 and 87; and in *parC* alterations are at codon 80 and 84, in the QRDR. For the *gyrB* and *parE*, mutations are much less common than those in *gyrA* or *parC*, and do not consistently result in detectable increase in the MIC (Morgan-Linnell et al., 2009). In a study performed in *E. coli*, Khodursky et al. demonstrated that the inhibition of topoisomerase IV became apparent only when gyrase was mutated during quinolone resistance (1995). Mutation in topoisomerase IV provided an additional 10-fold resistance to quinolones and prevented drug-induced catenane accumulation. The authors suggested that these differences might result from topoisomerase IV acting behind the replication fork, allowing repair of drug-induced lesions. Finally, concluding that the quinolone binding pockets of gyrase and topoisomerase IV are similar and substantial levels of drug resistance require mutations in both enzymes.

Amino acid substitutions in the primary enzyme target often constitute the prime step in the acquisition of resistance. Higher levels of resistance might be acquired by second

mutational steps, in which alterations occur in the secondary target enzyme. As the bacteria acquire further mutations, the primary target shifts between gyrase and topoisomerase IV and this switch is dependent on which enzyme is more sensitive to the antimicrobial agent. This pattern of stepwise mutations in alternating target enzymes affects the likelihood of selection of first-step resistant mutants (Hooper, 2001). In *E. coli,* it has been suggested that a single mutation in the gyrase decreases the fluoroquinolone susceptibility, so that topoisomerase IV becomes the target. Once the *parC* acquires mutation, *gyrA* again becomes the target (Morgan-Linnell et al., 2007).

Overexpression of the efflux pumps also confers low-level resistance against fluoroquinolones (Neyfakh et al., 1993; Pumbwe et al., 2006; Chang et al., 2007). This increase in efflux pump expression can be transient or stable (caused by mutations in genes regulating the expression of these efflux pumps) (Kern et al., 2000; Wang et al., 2001; Jumbe et al., 2003; Louie et al., 2007). In this mechanism, the drug is actively extruded from the cytoplasm across the periplasm and outer membrane to the cell exterior. The specific drug related features of a fluoroquinolone that determine whether it is affected by an efflux system are not defined fully but correlate with hydrophilicity of the compound (Piddock et al., 2002; Jumbe et al., 2003). Hydrophilic drugs such as ciprofloxacin are more likely to traverse the water filled channels as compared to relatively hydrophobic drugs such as levofloxacin. The hydrophilic character of ciprofloxacin makes it not only a better substrate but also a better inducer of the efflux

pumps (Louie et al., 2007). Thus, quinolones that are poor substrates for efflux pumps may carry an intrinsically lower risk for the development of antibiotic resistance.

Among other mechanisms of fluoroquinolone resistance that are not as prevalent as chromosomally-mediated mechanisms, are mutations in porins and plasmid acquisition (Hirai et al., 1986; Vila et al., 1999; Jacoby et al., 2003). In 1998, a plasmid-mediated quinolone resistance mechanism was first described to occur in a *Klebsiella pneumoniae* isolate from the United States (Martínez-Martínez et al., 1998). During the last decade, cases of plasmid-mediated quinolone resistance have increased (Jacoby et al., 2003). To date, three mechanisms have been described – *qnr, aac*(*6′*)-*Ib-cr, qepA*. The most common mechanism is acquisition of plasmid encoding for *qnr* genes (*qnrA, qnrS, qnrB*) (Wang et al., 2009; Strahilevitz et al., 2009). The mechanism of protective effect by the Qnr is not completely understood. In a study, Tran et al. showed through gel retardation assays that QnrA can bind to the DNA gyrase holoenzyme as well as to its respective subunits, GyrA and GyrB (2005). This binding occurred in the absence of relaxed DNA, ciprofloxacin, or ATP, indicating that the binding of QnrA to gyrase did not require the presence of the ternary complex of enzyme, DNA, and quinolone. Plasmids carrying *aac*(*6′*)-*Ib-cr* encode for an aminoglycoside acetyltransferase that inactivates aminoglycosides and fluoroquinolones through the acetylation of its piperazinyl substituent. Both, *oqxAB* and *qepA* encode for efflux pumps extruding quinolones (Hansen et al., 2004; Yamane et al., 2007; Zhao et al., 2010). *oqxAB* is a conjugative

plasmid conferring resistance to the antibiotic olaquindox, a quinoxaline derivative used in agriculture as a veterinary growth promoter, and was found in *E. coli* strains isolated from swine manure (Zhao et al., 2010).

Studies evaluating the effect of some *qnr* allele variants on quinolone MICs suggest that presence of *qnr* led to 2- to 32-fold increase in MICs of fluoroquinolones (Jacoby et al., 2006; Rodriguez-Martinez et al., 2008; Strahilevitz et al., 2009). In a study, Martínez-Martínez et al. transferred resistance between strains by conjugation and determined the frequency of fluoroquinolone resistant mutants in *E. coli* strains, with or without the quinolone resistance plasmid (1998). From a plasmid-containing *E. coli* strain, quinolone-resistant mutants could be obtained at more than 100 times the frequency of a plasmid-free strain, even though the MIC increase was only 16- to 32-fold.

2.1.4 Prevalence of fluoroquinolone resistance

In recent years, the extensive use of fluoroquinolones has contributed to a significant increase in resistance (Lautenbach et al., 2004; Boyd et al., 2008). In an analysis of *E. coli* clinical isolates, obtained between 1999 to 2004 (n ≅ 21,000) from a large county hospital, Boyd et al. suggested increase in the frequency of non-susceptibility to fluoroquinolones ($P \leq 0.01$) (Figure. 2.1) from ~6 to 25% over the period of time (2008). According to the European Antimicrobial Resistance Surveillance, prevalence of

fluoroquinolone resistance to *E. coli* has increased up to 50% in major parts of Europe (Kronvall, 2010). Similarly, another study with 35,790 isolates of Gram-negative pathogens show an increasing trend in fluoroquinolone resistance (Neuhauser et al., 2003). Figure 2.2 shows that the increasing rates of ciprofloxacin resistance correlated with the steadily increasing fluoroquinolone use ($r = 0.976$, $P<.001$ for *P. aeruginosa*; $r = 0.891$, $P = .007$ for Gram-negative bacilli; $r = 0.958$, $P<.001$) for the years of observation. Here Gram-negative bacilli included mainly *P. aeruginosa* (23%), Enterobacter species (14%), *Klebsiella pneumoniae* (14%), and *E. coli* (11%). The remaining 38% of isolates included Acinetobacter species (6%), *Serratia marcescens* (5%), *Stenotrophomonas maltophilia* (4%), *Proteus mirabilis* (4%), Citrobacter species (3%), and *Morganella morganii* (1%).

Figure 2.1: Non-susceptibility of fluoroquinolones (i.e., ciprofloxacin, gatifloxacin, levofloxacin, norfloxacin and ofloxacin) over time. The average percentage of resistant 'R' and intermediate 'I' isolates for all the tested fluoroquinolones combined for each year is shown. Adapted from Boyd et al., 2008.

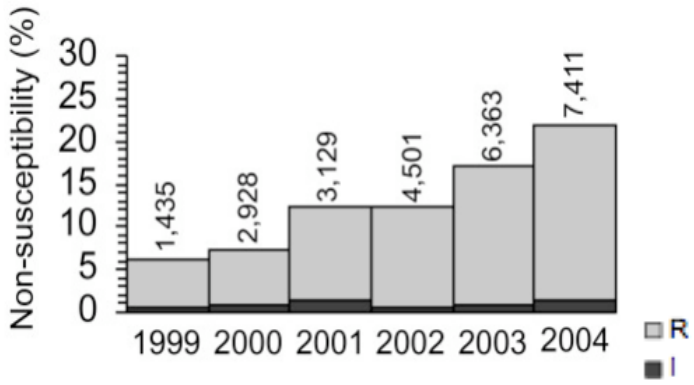
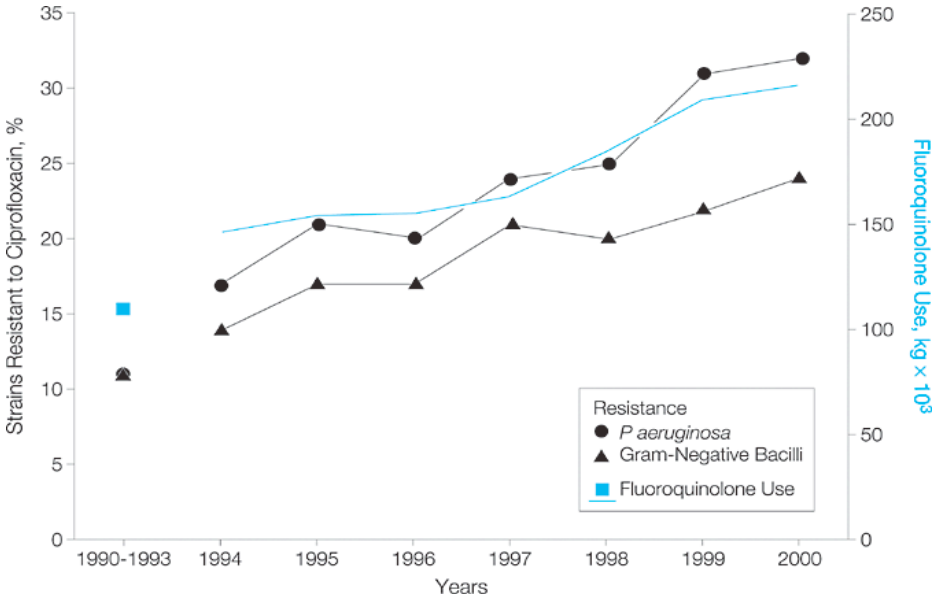


Figure 2.2: Fluoroquinolone use and resistance rates in *P. aeruginosa* and Gram-negative bacilli. Adapted from Neuhauser et al., 2003.



2.1.5 Levofloxacin

Levofloxacin is a broad-spectrum antibacterial agent with activity against Gram-positive, Gram-negative and atypical pathogens (Croom et al., 2003). Clinical efficacy of levofloxacin has been demonstrated in a range of infections such as urinary tract infections, skin and skin-structure infections, acute sinusitis, acute exacerbations of chronic bronchitis and community-acquired pneumonia. The long half-life of levofloxacin ($t_{1/2}$ ~ 7 h) allows for once-daily administration. Pharmacokinetics of levofloxacin is linear over the dose range 500 - 1000 mg once daily for multiple-dose administration. Our studies were performed mainly using levofloxacin because it is widely used in the clinic and usage of levofloxacin has been associated with lower potential to select resistant mutants (Linde et al., 2004; Jumbe et al., 2003; Louie et al., 2007).

Similar to other fluoroquinolones, levofloxacin is a concentration-dependent antibacterial agent. Therefore, the ratio of area under the plasma concentration-time curve over 24 h at steady state (AUC) or maximum plasma concentration (C_{max}) to MIC for unbound drug can be used as a predictor of microbiological and clinical efficacy (Preston et al., 1998). Studies suggest that dose exposures that achieve a C_{max}/MIC ratio >10 and an AUC/MIC ratio >100-125, are associated with maximal bacterial eradication in Gram-negative

bacteria. In Gram-positive bacteria such as *S. pneumoniae*, an AUC/MIC ratio >30 has been associated with clinical eradication (Nightingale et al., 2000; Zhanel et al., 2002). With AUC/MIC ratio ≥ 35, levofloxacin was bactericidal *in vitro* against both levofloxacin-susceptible and -resistant strains of *S. pneumoniae* (Ibrahim et al., 2002).

2.2 ESCHERICHIA COLI

2.2.1 Introduction

E. coli is a Gram-negative, non-spore-forming, facultative microorganism. It is the main aerobic commensal bacterial species in the gastrointestinal tract.

E. coli can cause several intestinal and extra-intestinal infections such as urinary tract infections, meningitis, peritonitis, mastitis, septicemia and pneumonia. It is the leading cause of urinary tract infections (UTIs) and intra-abdominal infections (Rossi et al., 2004). UTIs are one of the most common infectious diseases diagnosed in outpatients as well as inpatients. *E. coli* is the most frequent pathogen isolated worldwide from these UTIs and accounts for 50% of the cases (Gaynes et al., 2005). *E. coli* has also been commonly associated with nosocomial or community-acquired bacteremia and represents approximately 15% of cases (Luzzaro et al., 2002; Wisplinghoff et al., 2004). Some problematic cases of *E. coli* pyomyositis have emerged among patients with hematologic malignancy. This is typically caused by *E. coli*-ST131 strain, which has been implicated

in fluoroquinolone-resistant, ESBL-positive infections worldwide (Vigil et al., 2010).

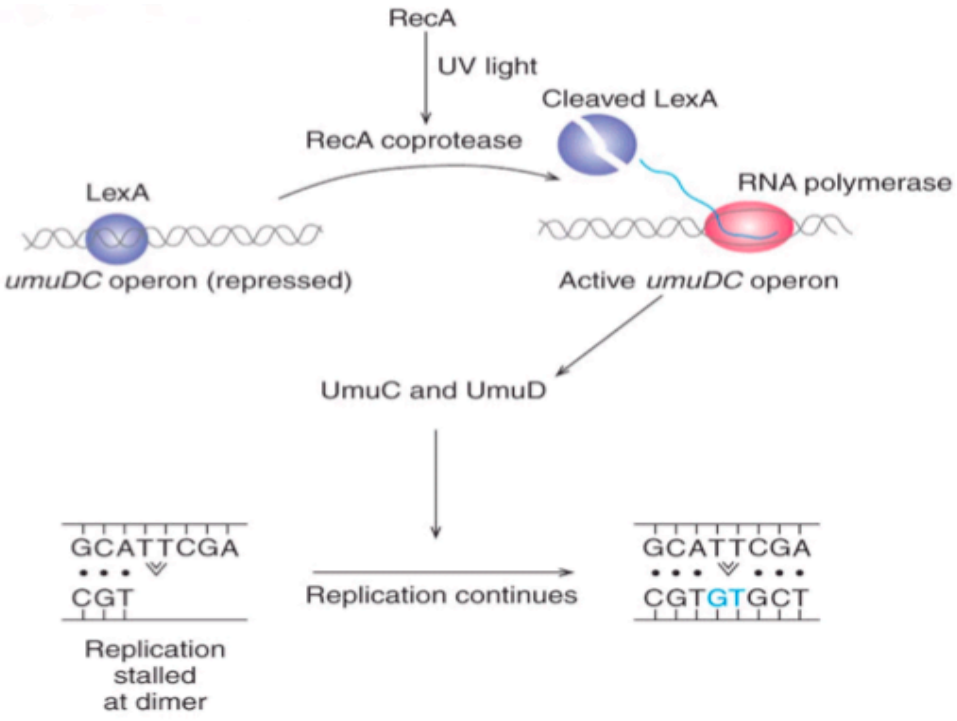
2.2.2 SOS response

Lately, studies have suggested that bacteria also play an active role in inducing mutations in the event of exposure to certain classes of antibiotics or other environmental stress(es). One of the mechanisms by which these mutations are induced is the SOS response system (Cirz et al., 2005). The SOS system is a global response to DNA damage leading to the induction of DNA repair and enhanced mutagenesis. A central part of the SOS response is the derepression of various SOS genes, which are under the direct and indirect transcriptional control of a repressor – LexA (Erill et al., 2007). Another important protein involved in regulating the SOS response is RecA (the sensor of the system). RecA is a recombinase protein that has coprotease activity, functions in DNA strand exchange and facilitates replicative bypass of DNA lesions (Ogawa et al., 1990). In a non-triggered state, LexA binds as a dimer in the promoter region of SOS genes and down-regulates its own expression and that of other SOS genes. Formation of single-stranded DNA or a stalled replication fork resulting from DNA damage acts as a trigger for the SOS system and activates RecA. Upon activation, coprotease activity of RecA causes autocatalytic cleavage of the LexA dimer and leads to derepression of the SOS genes. The LexA regulon in most cases includes recombination and repair genes - *recA*, *recN*, and *ruvAB*; nucleotide excision repair genes - *uvrAB* and *uvrD*; the error-prone DNA polymerase (pol) genes - *dinB* (encoding pol IV), *umuDC* (encoding pol V) and DNA polymerase II,

along with other genes. Induction of the SOS response initiates several processes such as cell division inhibition, excision repair, up-regulation of tri-carboxylic acid cycle and error prone replication; hence trading long term fidelity with short term viability (Figure 2.3). Overall, this process leads to a higher rate of mutant formation.

Despite the SOS system being a global stress response system, there is diversity among various bacterial species. While a common homogeneity between most of the bacterial species is the presence of RecA, LexA and a tightly controlled error prone DNA polymerase, the number of genes controlled by the LexA regulon differ among bacterial species. LexA regulon controls 16 genes in *S. aureus* and 43 genes in *E. coli* (Cirz et al., 2005; Cirz et al., 2007). In our study, we found difference in the resistance emergence pattern when *recA* was inhibited in these two strains, which could be linked to the difference in the number of genes regulated by this operon in these strains (Singh et al., 2010).

Figure 2.3: Schematic depicting induction of the SOS response system in bacteria by UV light. The *umuDC* gene in *E. coli* encodes for an error prone polymerase, hence leading to mutations (Source: http://cms.daegu.ac.kr/sgpark/molecularbiology/DNA.htm).



2.2.3 Efflux pumps in *E. coli*

Introduction

Bacterial genome sequence analyses have revealed that 5 - 10% of the total encoded genes are transport proteins. Drug efflux pumps constitute 6 - 18% of all transporters, indicating the enormous capabilities of bacteria to combat toxic insult by antimicrobial

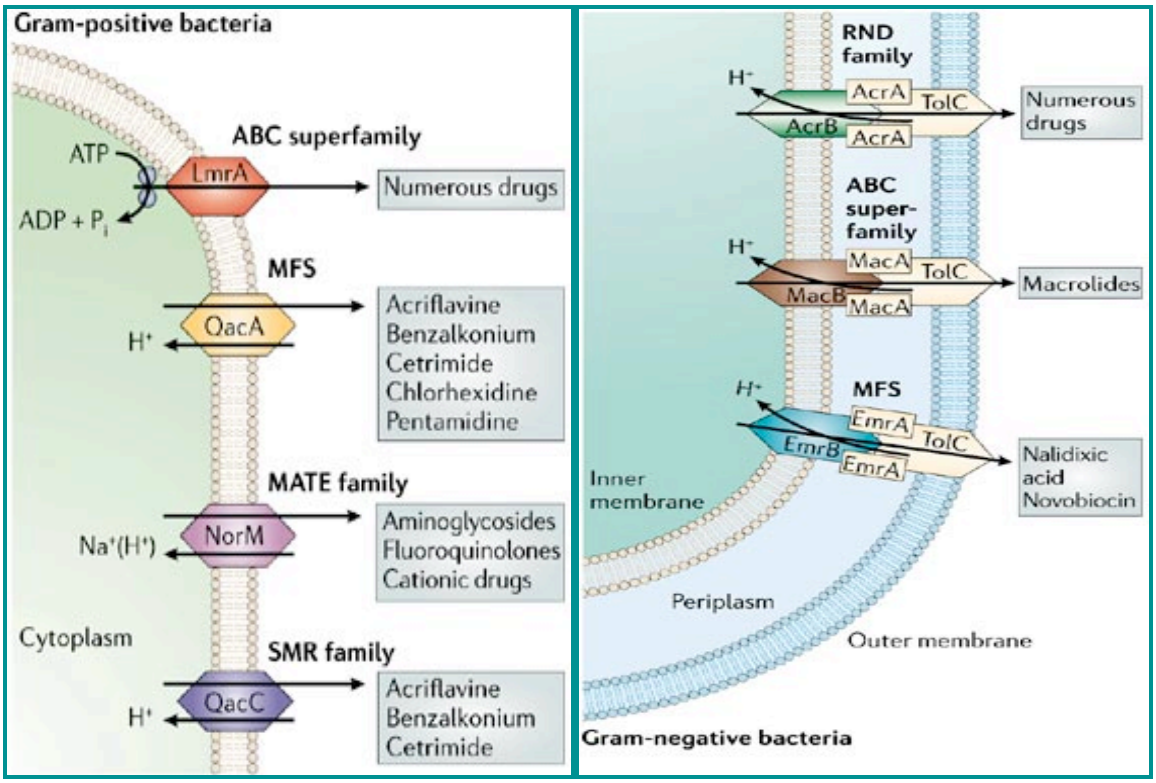
agents. Five families of bacterial drug efflux pumps have been identified until now (Paulsen et al., 1998). Transport can either be driven by ATP hydrolysis, as in the case of the ATP-binding cassette (ABC), or by utilizing proton motive force (PMF). The four PMF-dependent families are Small Multidrug Resistance Family (SMR), Major Facilitator Superfamily (MFS), Multidrug and Toxic Compound Extrusion Family (MATE), and Resistance/Nodulation/Cell Division Family (RND) (Nikaido et al., 2001).

Efflux pumps, belonging to the RND family, extrude a large variety of structurally diverse compounds and therefore are called multidrug resistant (MDR) transporters. Genes encoding MDR pumps are normal constituents of bacterial chromosomes and increased antibiotic resistance is a consequence of overexpression of these pumps. All efflux pumps in Gram-positive bacteria and some in Gram-negative bacteria pump out their substrates across a single cytoplasmic membrane (Figure 2.4). Other efflux pumps from Gram-negative bacteria efflux substrates directly into the external medium, bypassing the outer membrane and the periplasm. These pumps are organized in complex three component structures, which traverse both inner and outer membranes such as AcrAB-TolC efflux pump (Nikaido et al., 2001; Pos, 2009). This structural organization makes efflux action in Gram-negative bacteria much more robust than Gram-positive bacteria, since substrates are extruded from the cytoplasm as well as periplasm, directly into the external medium. This phenomenon is referred to as trans-envelope efflux (Lomovskaya et al., 2007). This structural organization combined with the MDR

characteristics of RND efflux pumps makes them difficult to overcome by pharmacological interventions.

E. coli has more than 40 putative efflux pumps, out of which fluoroquinolones are substrates of three efflux pumps- AcrAB-TolC, MdfA and NorE. The AcrAB-TolC efflux pump is a member of the RND family, while MdfA and NorE belong to the MFS and MATE families, respectively (Nikaido et al., 2001). AcrAB-TolC has been suggested to be the most important efflux pump in *E. coli*. Yang et al. compared the contribution of these three efflux pumps to quinolone MIC changes in *E. coli* (2003). They suggested that overproduction of each of these three pumps separately resulted in roughly similar levels of quinolone resistance. A synergy in quinolone resistance was shown when *acrAB* was overexpressed simultaneously with *norE* or *mdfA*. Deletion of *acrAB* alone and all of the three pumps together had the same effect on the susceptibility of fluoroquinolones. The authors also found that the strain with *acrAB* deletion were the most susceptible when compared to mutants strains with deletion in *norE* and/or *mdfA.* The maximum quinolone resistance mediated by efflux pumps was suggested to be ~10-fold, irrespective of any increase in production of these pumps.

Figure 2.4: Schematic showing important efflux pumps in Gram-positive (left) and Gram-negative (right) bacteria. Adapted from Piddock, 2006a.



Gram-positive bacteria Gram-negative bacteria

AcrAB-TolC efflux pump

The AcrAB-TolC efflux pump functions as a tripartite complex, consisting of AcrA as the membrane fusion protein, AcrB as the cytoplasmic membrane multidrug pump component and TolC as the outer membrane channel protein (Nikaido et al., 2001). The AcrB is the main pump component capturing substrates, pumping them out, and is composed of 12 trans-membrane domain and an unusually large periplasmic domain (Piddock, 2006b). This MDR efflux pump system confers resistance to a diverse range of compounds, such as dyes, detergents, fluoroquinolones, β-lactams, chloramphenicol, erythromycin and tetracycline.

In 1995, a study by Ma et al. demonstrated that deletion of *acrAB* increased susceptibility of *E. coli* to cephalothin and cephaloridine, but the permeability of these agents across the outer membrane was not increased suggesting *acrAB* coded for a multidrug efflux pump (1995). The natural environment of an enteric bacterium such as *E. coli* is enriched in bile salts and fatty acids. An *acrAB* deleted mutant was found to be hypersusceptible to bile salts and to decanoate. In addition, *acrAB* expression was elevated by growth in 5 mM decanoate. These results suggested that one major physiological function of AcrAB was to protect *E. coli* against these and other hydrophobic inhibitors. Transcription of *acrAB* was increased by other stress conditions including 4% ethanol, 0.5 M NaCl, and stationary phase in Luria-Bertani medium. They also showed that *acrAB* expression was increased in *mar* (multiple antibiotic resistant) mutants. A systematic analyses of MIC

changes using *mar* and *acrAB* knockout strains by Okusu et al. further suggested that *marR* (global repressor of *acrAB*) mutation was incapable of increasing the resistance level in the absence of the AcrAB efflux pump (1995). Thus, AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of *E. coli mar* mutants. A study done by Oethinger et al. in fluoroquinolone-resistant mutants, selected from a wild-type *E. coli* strain by exposure to increasing levels of ofloxacin on solid medium, suggested AcrAB is the major efflux pump (2000). They showed that inactivation of the *acrAB* locus made all strains, including those with target gene mutations, hypersusceptible to fluoroquinolones. This study indicated that in the absence of the AcrAB efflux pump, gyrase mutations failed to produce clinically relevant levels of fluoroquinolone resistance.

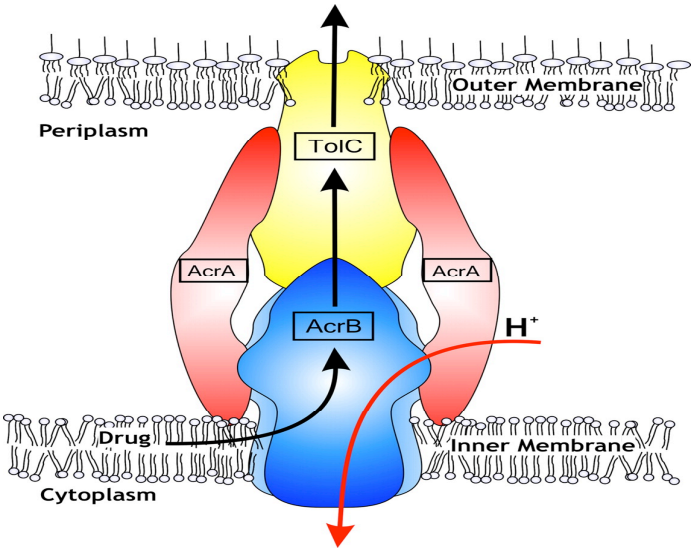


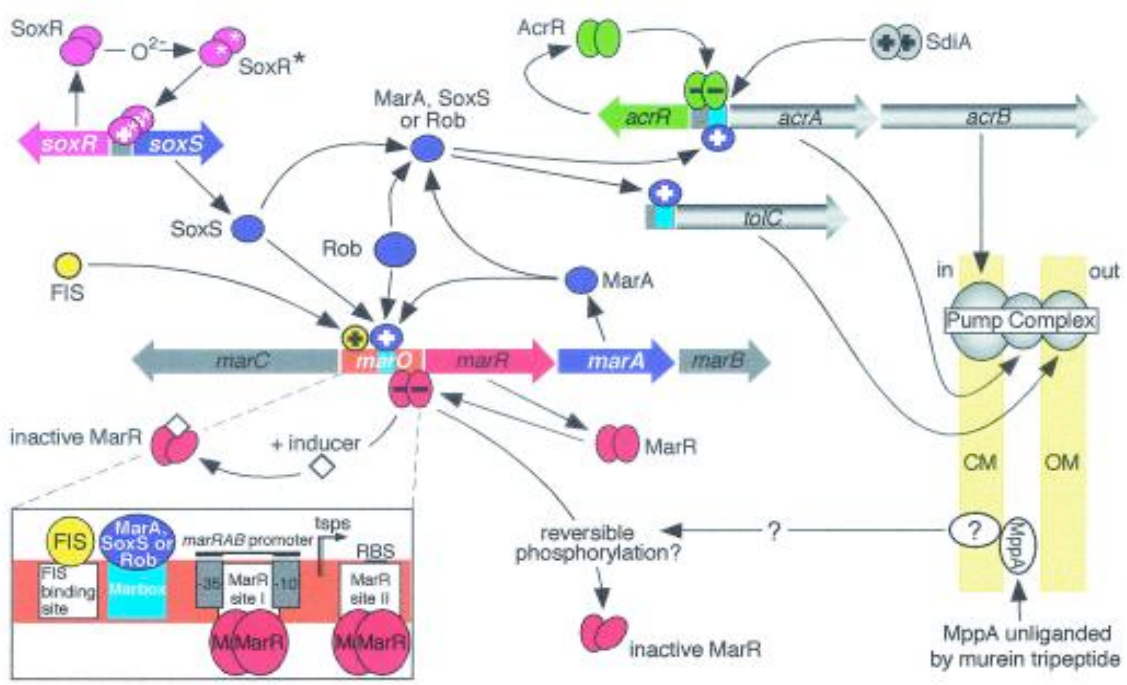
Figure 2.5: Schematic showing a typical Gram-negative cell envelope along with tripartite complex of the AcrAB-TolC efflux pump. Adapted from Pos, 2009.

Regulation of AcrAB efflux pump

The genes *acrAB* form an operon (Figure 2.6). The local dimeric repressor protein AcrR prevents excessive production of AcrA and AcrB, whereas a regulatory protein involved in cell division - SdiA can increase AcrAB expression (Ma et al., 1995; Ma et al., 1996; Rahmati et al., 2002). Wang et al. demonstrated that mutations in *acrR* contributed to high-level fluoroquinolone resistance in clinical isolates of *E. coli* (2001).

The *mar* regulatory locus, which consists of the *marRAB* operon, also appears to play a critical role in the global regulation of AcrAB expression. The first gene of this operon – *marR*, represses the expression of its own gene and the two others constituting the *marRAB* operon. Thus, it controls the intracellular level of global activator MarA (Keeney et al., 2008). Binding of inducing compounds or phosphorylation transforms MarR into a non-DNA-binding conformation, thereby permitting *marRAB* transcription to proceed. The increased intracellular levels of MarA can then bind adjacent to the promoters of *mar* regulon genes, such as *acrAB* and *tolC*, and activate their transcription. Some other MarA homologs such as SoxS and Rob can also activate *marRAB* transcription.

Figure 2.6: Schematic representing the transcriptional regulation of AcrAB efflux pump. Adapted from Grkovic et al., 2002.



2.3 IN VITRO INFECTION MODELS

2.3.1 Introduction

One of the important reasons for antibiotic resistance is that the dose–response relationships are not well known and the dosing regimens of antibiotics are often not optimal. The effect of drug concentration on bacteria, i.e. the pharmacodynamics (PD),

and the ability of the drug to reach its target or pharmacokinetic (PK) properties, determine the response to the antibacterial treatment. By linking the concentration–time course (at the site of action) to the drug effect (PK/PD), potentially effective dosing strategies that maximize the likelihood of clinical response can be identified earlier in drug development (Drusano et al., 2004; Levison et al., 2009).

Both, bacterial growth and killing under antibiotic exposure need to be evaluated to fully characterize the PD of the antibiotic. Since these are difficult to measure in human tissue, animal and *in vitro* models have been developed (Hickey, 2007). While animal models can imitate the human infection milieu more closely than *in vitro* models, a major disadvantage of animal models is differences in the PK (i.e. faster clearance and difference in metabolism). These factors limit or necessitate complicated scaling methods for extrapolating data from animals to humans. Conventional susceptibility testing methods (e.g. minimum bactericidal concentration (MBC) and MIC) have discrete endpoints. These endpoint measurements do not provide any information regarding the rate and extent of bacterial killing (PD changes) during the incubation period. Another limitation of these susceptibility tests is that these methods employ constant antibiotic concentrations; which is in contrast to the continuously changing concentrations observed *in vivo*. To tackle these problems, various *in vitro* PK/PD models have been developed. Taking into consideration various PK determinants, such models allow a more comprehensive study of the PD effects demonstrated by antibiotics.

2.3.2 Static versus dynamic models

The two main characteristics of *in vitro* models are drug exposure and bacterial burden. Hence, *in vitro* models are characterized in two main categories: (1) Static models, where there is no replacement or change of the media, hence constant drug concentration is present (2) Dynamics models, where the media is constantly flowing and fluctuating drug concentration is obtained.

In static models, bacteria are suspended homogeneously in a culture vessel with constant antibiotic concentration in the media. All conditions remain the same over the entire observation period and the changes in bacterial population can be studied over time. In the dynamic models, the main idea is to simulate the body clearance or half-life of the antibiotic by changing drug concentrations (Mueller et al., 2004). In dynamic models, the drug concentration in the culture vessel can be changed via substitution with fresh media or by simple dilution. Fresh media is pumped from a reservoir into the culture vessels and from there into the waste. Substitution means removal of a defined volume from the *in vitro* model and replacing the volume with fresh media. In this case, both inflow and outflow are controlled. The volume in the model remains constant. Dilution means addition of a defined volume of media to the culture vessel at regular intervals. Hence, the drug concentration in the culture vessel is diluted. The input of media in dilution

models can occur continuously or stepwise. The input of the drug can mimic bolus, infusion or first-order absorption pharmacokinetics (Gloede et al., 2010).

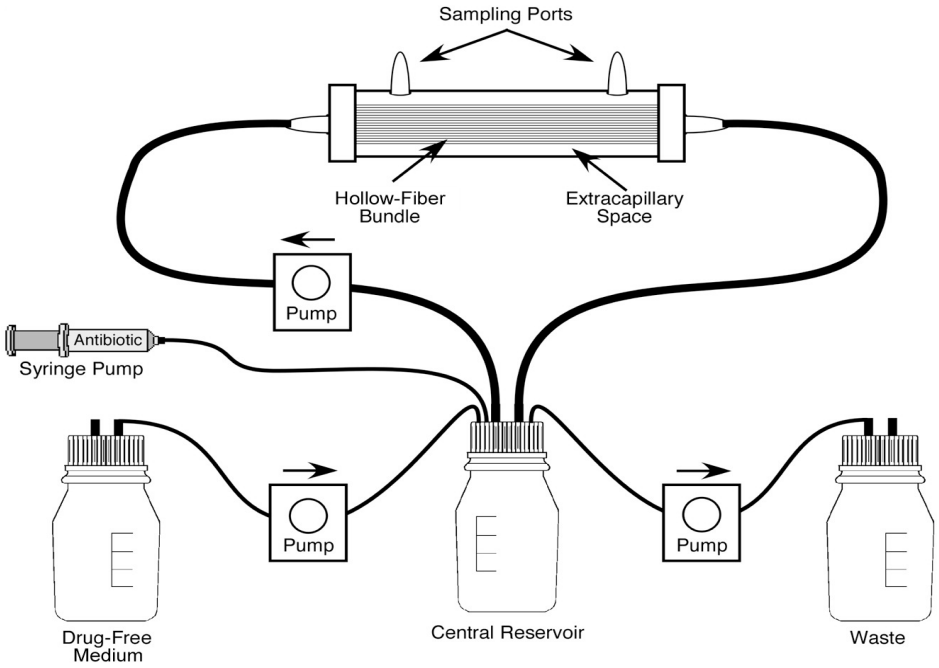
Another approach for dynamic *in vitro* models is via drug diffusion across a membrane (dialysis) where the concentration gradient is the driving force. The dialysis model consists of a central compartment, where the drug initially appears after dosing, and another compartment with bacteria, which is separated by a semi-permeable membrane (permeable for drug and media but not for bacteria). Fresh media is continuously pumped from a reservoir into the central compartment and then into the waste (Blaser et al., 1985).

While in static models there is no loss of bacteria, the dynamic models by virtue of having a flowing media may or may not result in bacterial loss. The bacterial burden represents the magnitude of the PD effect. Loss of bacteria observed in some models can have a substantial influence on the results. Hence, some of the dynamic models have been modified, by attaching a filter to minimize bacterial loss. To define the loss of bacteria in an *in vitro* model the terms open and closed are used: open models allow the exchange of bacteria with the environment; and closed models have no bacterial exchange (Murakawa et al., 1980).

2.3.3 Hollow fiber Infection model

The hollow fiber infection model (HFIM) is an *in vitro* dynamic model (dialysis) that simulates fluctuating drug concentration. A schematic diagram of the HFIM is shown in Figure 2.7. The main component of this model is the hollow fiber (HF) cartridge. These hollow fiber cartridges consist of bundles of HF capillaries housed in a plastic casing. The fibers have numerous pores that permit the passage of nutrients and low molecular weight substances such as antibiotics, but exclude bacteria. The antibacterial agent is administered into the central reservoir by a syringe and this antibiotic containing media is continuously pumped through the HF cartridge by means of an internal circulatory pump. Thus, microorganisms inoculated in the extracapillary space of the hollow fiber cartridge, are exposed to conditions approximately those prevailing in the central reservoir. The antibiotic containing media is iso-volumetrically replaced with drug-free media, simulating the half-life of the drug. The rate constant of elimination of drug is the rate of fresh media infusion divided by the volume of the media in the total system. These systems can be modified to simulate either a one-compartment or two-compartment model with exponential elimination.

Figure 2.7: Schematic of the hollow fiber infection model. Adapted from Tam et al., 2007.



2.3.4 Advantages and disadvantages of *in vitro* infection models

Advantages

1. HFIM enables simulation of fluctuating drug concentrations and hence can closely mimic the dynamics of interaction between the drug and microorganisms compared to other *in vitro* models, which use static drug concentrations.

2. In contrast to animal models, *in vitro* models can mimic human PK more closely and are thus better suited for the investigation of antimicrobial activity.
3. HFIM is more flexible and adaptable to different conditions such as a higher inoculum and for longer duration as compared to animal models.
4. Homogenous collectives (population) are possible in these systems as compared to human subjects where usually a heterogenous population exists.
5. Flexibility in dosing schedule and designs enable easy investigation of the PK/PD index relating to the microbiological outcome.
6. PK can be applied directly and the time course of an antimicrobial agent can be monitored precisely.
7. HFIM allows better study of antimicrobial resistance because a higher bacterial burden can be used in these systems and hence chances of detecting mutants are higher.
8. Bacterial eradication can be evaluated in a time dependent way so that rate of kill can be studied.
9. The drug administration scheme can be varied more easily as compared to human subjects.
10. They allow determination of time–kill behavior and the optimization of dosing regimens and breakpoints.

Disadvantages

1. The pathology of infection, virulence and metabolic behavior of pathogen cannot be studied.
2. The derived PD parameter cannot be directly transferred to *in vivo* because of lack of an immune component and the complexity present *in vivo.*
3. These models need special conditions, such as a temperature-controlled environment and there is risk of contamination.
4. There is a difference in the growth environment between the *in vivo* and *in vitro* conditions. This may lead to phenotypic differences in the bacteria. Bacterial growth might be faster in case of *in vitro* systems leading to stronger competition for nutrients. This may lead to a higher production of antimicrobial drug targets, resulting in a higher susceptibility *in vitro.*

CHAPTER 3

Dosing and Resistance Selection in a High Inoculum of *Escherichia coli* with Pre-existing Mutants

This chapter has been published: Singh et al., 2009

3.1 ABSTRACT

Escherichia coli is the leading bacterium implicated in intra-abdominal infections. In these infections a high bacterial burden with pre-existing resistant mutants are likely to be encountered and resistance could be amplified with sub-optimal dosing. Our objective was to investigate the pharmacodynamics of moxifloxacin against a high inoculum of *E. coli* using a HFIM. Three wild-type strains of *E. coli* (ATCC 25922, MG1655 and EC28044) were studied; approximately $2x10^8$ CFU/ml were exposed to escalating dosing regimens of moxifloxacin (ranged from 30 mg to 400 mg; q24h). Serial samples were obtained from HFIM over 120 h to enumerate the total and resistant sub-population. Quinolone resistance determining regions of *gyrA* and *parC* of resistant isolates were sequenced to confirm the mechanism of resistance. Pre-exposure MIC of the three wild-type strains was 0.0625 mg/l. Simulated moxifloxacin concentration profiles in HFIM was satisfactory (r^2 ≥0.94). Placebo experiments revealed natural mutants but no resistance amplification. Regrowth and resistance amplification was observed between 30 mg (AUC/MIC = 47) and 80 mg dose (AUC/MIC = 117). Sustained bacterial suppression

was achieved at and above 120 mg dose (AUC/MIC = 180). Point mutations in *gyrA* (D87G or S83L) were detected in resistant isolates. Our results suggest that sub-optimal dosing may facilitate resistance amplification in a high inoculum of *E. coli*. The clinical dose of moxifloxacin (400 mg q24h) was adequate to suppress resistance development in three wild-type strains. Clinical relevance of these findings warrants further *in vivo* investigation.

Keywords: moxifloxacin, quinolone, *E. coli*, resistance

3.2 INTRODUCTION

Moxifloxacin is a broad-spectrum fluoroquinolone used to treat a variety of infections including complicated intra-abdominal infections and acute bacterial sinusitis. However, recent increase in resistance to fluoroquinolones is a major concern and warrants for immediate steps to be taken to suppress (or delay) emergence of resistance (Lautenbach et al., 2004; Boyd et al., 2008). One of the strategies to maximize microbial kill and to suppress emergence of resistance is pharmacodynamic-based dosing. It is widely accepted that a dense bacterial population consists of two sub-populations - susceptible and resistant (Blondeau et al., 2001). Considering the mutational frequency of bacteria, a high inoculum of bacteria (more than the inverse of mutational frequency to resistance) is likely to harbor resistant mutants at the start of therapy and a sub-optimal dose exposure would selectively amplify the resistant sub-population. On the other hand, an optimal dose exposure could lead to suppression of both the susceptible and resistant sub-population.

Escherichia coli is a common pathogen implicated in intra-abdominal infections (Rossi et al., 2006), which are often associated with high bacterial inocula (Konig et al., 1998). Pre-existing resistant mutants are likely present in these infections and a sub-optimal dose exposure will lead to emergence of resistance. Several studies have

examined the pharmacodynamics of fluoroquinolones (e.g. ciprofloxacin, levofloxacin and moxifloxacin), but none has investigated the propensity of resistance suppression by moxifloxacin against a high inoculum of *E. coli* (Zinner et al., 2004; Scaglione et al., 2003; Firsov et al., 2000; LaPlante et al., 2007).

Resistance to fluoroquinolones could be caused by multiple mechanisms. Chromosomally-mediated resistance is more common and may occur through alterations in the genes encoding DNA gyrase and topoisomerase, where fluoroquinolones target binding sites are located (Broskey et al., 2000; Morgan-Linnell et al., 2009). Mutations are most commonly detected in the region called 'quinolone resistance determining region' (QRDR), which is present in both subunits of DNA gyrase (*gyrA* and *gyrB*) and topoisomerase (*parC* and *parE*). Resistance can also occur by overexpression of efflux pumps such as AcrAB (Broskey et al., 2000; Morgan-Linnell et al., 2009). Lately, plasmid-mediated quinolone resistance (such as *qnrA*) is also known to be implicated (Morgan-Linnell et al., 2009; Cattoir et al., 2009).

In this study, we utilized a HFIM to study the pharmacodynamics of moxifloxacin against a high inoculum of *E. coli.* Specifically, the relationship between drug exposure and resistance amplification was examined. Additionally, we also attempted to characterize the mechanism of moxifloxacin resistance developed as a consequence of sub-optimal pharmacodynamic exposure.

3.3 MATERIALS AND METHODS

3.3.1 Antimicrobial agent

Moxifloxacin powder was a gift from Bayer Pharmaceuticals (West Haven, CT). A stock solution of moxifloxacin in sterile water was prepared, aliquoted and stored at -70 °C. Prior to each susceptibility testing, an aliquot of the drug was thawed and diluted to the desired concentrations with cation-adjusted Mueller-Hinton broth (Ca-MHB) (BBL, Sparks, MD) or sterile water, as required.

3.3.2 Microorganisms

Three wild-type strains of *E. coli* - American type culture collection (ATCC) (Manassas, VA) 25922, MG1655 and EC28044 were used in this study. MG1655 is a molecular standard strain, which the whole genome has been sequenced (Blattner et al., 1997). EC28044 is a urinary (pathogenic) isolate obtained from a patient in Houston, TX. These isolates were stored at -70 °C in Protect® storage vials (Key Scientific Products, Round Rock, TX). Fresh isolates were sub-cultured at least twice on 5% blood agar plates (Hardy Diagnostics, Santa Maria, CA) for 24 h at 35 °C prior to each experiment.

3.3.3 Susceptibility studies

MICs / minimum bactericidal concentrations (MBCs) were determined in Ca-MHB using the broth macrodilution method as described by the Clinical and Laboratory Standards Institute (CLSI, 2007*)*. The final concentration of bacteria in each broth macrodilution tube was approximately 5 x 10^5 CFU/ml of Ca-MHB. Serial two-fold dilutions of moxifloxacin were prepared. The MIC was defined as the lowest concentration of drug that resulted in no visible growth after 24 h of incubation at 35 °C. Samples (50 μl) from clear tubes and the cloudy tube with the highest drug concentration were plated on Mueller-Hinton agar II (MHA) plates (Hardy Diagnostics, Santa Maria, CA) to determine MBC. The MBC was defined as the lowest concentration of drug that resulted in ≥ 99.9% kill of the initial inoculum. Drug carryover effect was assessed by visual inspection of the distribution of colonies on MHA plates. The experiment was repeated at least twice on separate days.

3.3.4 Hollow fiber infection model studies

The basic design of the system has been described previously (Tam et al., 2007). Human unbound pharmacokinetic exposures of moxifloxacin were simulated over five days with repeated doses given once daily. The targeted elimination half-life was 12 h (reported range 7 to 14 h) (Simon et al., 2003; Fuhrmann et al., 2004; Stass et al., 2001).

Various dose exposures ranging between 30 mg to 400 mg given once daily were simulated.

On the day of experiment, overnight culture of the isolate was diluted with pre-warmed Ca-MHB and incubated further at 35 °C until reaching late log-phase growth. The targeted inoculum was calculated based on absorbance at 630 nm and 20 ml of approximately 2 x 10^8 CFU/ml bacteria were introduced to the extracapillary space of the hollow fiber cartridge (Fibercell systems, Inc., Frederick, MD). The experimental set up was maintained at 35 °C in a humidified incubator for 120 h. Bacterial samples (500 µl) were taken at various time points (0, 4, 8, 24, 48, 72, 96, 120 h) (pre-dose, where appropriate) in duplicate from the sampling ports. Before plating, the samples were centrifuged at 10,000 g for 15 min at 4 °C. Supernatant was discarded and the pellet was re-suspended with saline to minimize the drug carryover effect. These samples (50 µl) were spirally plated (Spiral Biotech, Bethesda, MD) on drug-free MHA plates and moxifloxacin- supplemented MHA plates. Plating on drug-free MHA plates was to quantify the total bacterial population and the moxifloxacin-supplemented plates (3x MIC) were to ascertain the bacterial population with reduced susceptibility (resistance). Considering the fact that two-fold difference in MIC value is a generally accepted interday deviation for MIC testing, a 3x MIC supplemented plate would allow reliable detection of the resistant population. Drug-free plates were incubated for 24 h and moxifloxacin-supplemented plates were incubated up to 72 h (if required) at 35 °C before

counting. Bacterial density was quantified by an automated colony counter (IUL, Farmingdale, NY). The theoretical lower limit of detection was 400 CFU/ml. Placebo HFIM experiments were performed for the three wild-type strains. Samples (500 μl) were also collected in duplicate on alternative days at various time points to ascertain the simulated drug exposure. These samples were stored at -20 °C until analyzed (not exceeding one month) by a validated HPLC assay as described below. A one-compartment linear model was fit to the observed concentration-time profiles using the ADAPT II program (D'Argenio et al., 1997).

3.3.5 High-performance liquid chromatography assay

A validated high-performance liquid chromatography (HPLC) method (Waters 2695 separations module) was used to determine the concentration of moxifloxacin in samples obtained from HFIM. The column used was NovaPak C18 [3.9 X 15 mm, particle size 4 μm (Waters Corporation, Milford, MA)]. A C18 guard column (Waters) was also used. Levofloxacin (100 mg/l) was utilized as an internal standard. The mobile phase included acetonitrile, 0.1 M phosphoric acid (adjusted to pH 3) and 0.01 M N-octylamine (adjusted to pH 3). A gradient elution was performed. Detection was done at 290 nm by a UV detector (Waters 2487 UV detector). Stock solution (1 g/l) of moxifloxacin and levofloxacin was prepared in HPLC grade water and stored at -20 °C until used. Working solution (100 mg/l) of moxifloxacin was prepared by spiking the

stock solution into Ca-MHB. Aliquots of 200 µl of standards or samples were pipetted into 1.5 ml snap-cap polypropylene eppendorf tubes and 20 µl of internal standard solution (100 mg/l) added into each tube. The tubes were vortexed for 30 seconds. 200 µl of the solution was transferred to sample vial inserts for injection onto the HPLC column. The retention time for moxifloxacin was 6.3 min and for levofloxacin (internal standard) was 5.4 min. The assay was linear over the range of 0.0625 mg/l to 20 mg/l ($r^2 \geq 0.996$). The intraday and interday coefficient of variation for the assay was 5% and 7%, respectively.

3.3.6 Confirmation of moxifloxacin resistance mechanisms

Six random isolates were recovered from the moxifloxacin-supplemented plates at the end of the each HFIM experiment and sub-cultured on 5% blood agar plates. Susceptibilities of these recovered isolates to moxifloxacin were repeated to confirm the emergence of resistance. In addition, E-test (AB Biodisk, Piscataway, NJ) was also performed to evaluate cross-resistance to ciprofloxacin and levofloxacin. Subsequently, the quinolone resistance determining region (QRDR) of the *gyrA* and *parC* genes of resistant isolates (and their respective parent strains) were amplified by PCR, to provide a molecular evidence of moxifloxacin resistance. The genebank accession numbers and sequences of primers used are listed in Table A of the Appendix. Amplification was performed in a thermal cycler (Applied Biosystems, Foster City, CA) with an initial

denaturing step of 94 °C for 10 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, and final extension at 72 °C for 10 min. No template (negative) controls were included and the reactions were evaluated by electrophoresis in 2% agarose gel. The PCR products were sequenced by Lonestar laboratories, Houston, TX.

3.4 RESULTS

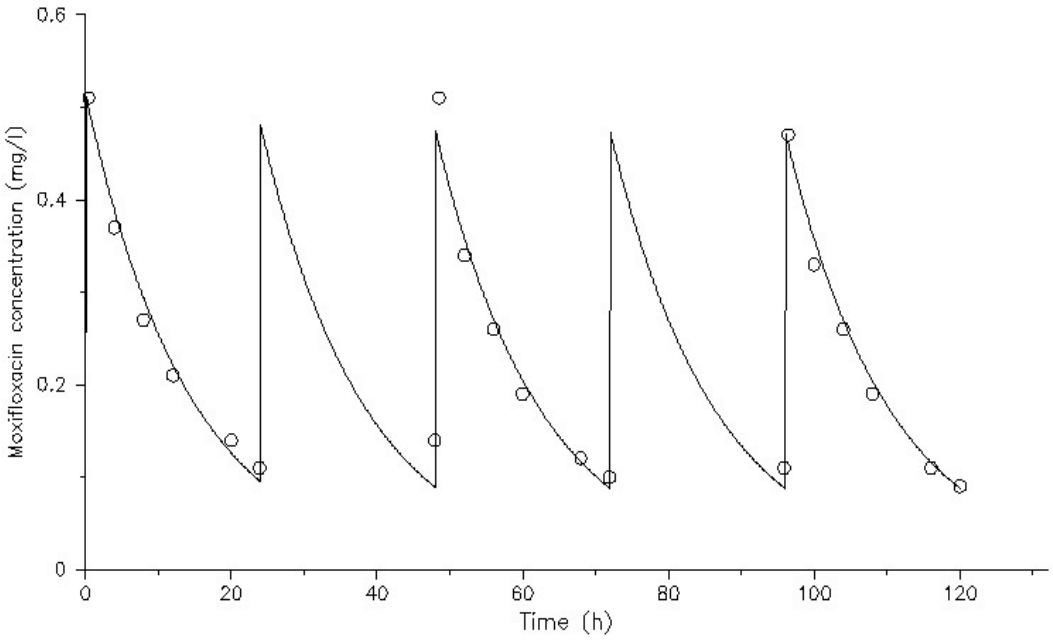
3.4.1 Susceptibility studies

The pre-exposure MIC of the three wild-type strains of *E. coli* was 0.0625 mg/l. The pre-exposure MBC for ATCC 25922 and MG1655 was also 0.0625 mg/l, while that for EC28044 was 0.125 mg/l.

3.4.2 Pharmacokinetic validation

Satisfactory simulated concentrations ($r^2 \geq 0.94$) were achieved in all the HFIM experiments. A typical concentration-time profile with the best-fit model is shown in Figure 3.1.

Figure 3.1: A typical simulated concentration- time profile of moxifloxacin over 5 days.



Moxifloxacin was administered once daily to maintain a target C_{max} of 0.5 mg/l. Open circles represent experimental observations and the solid line represent the best-fit model ($r^2 = 0.976$).

3.4.3 Hollow fiber infection model studies

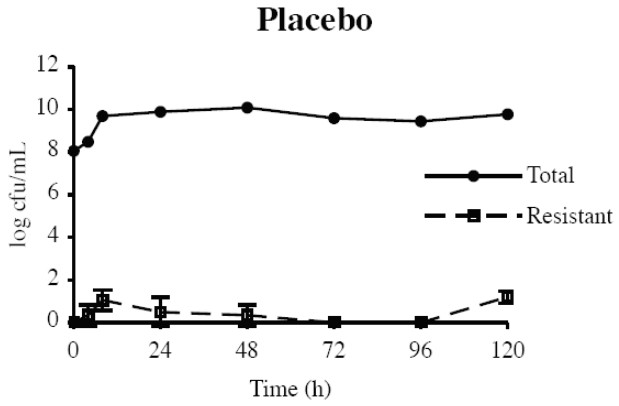
The changes in bacterial burden over time with different dosing regimens of moxifloxacin are shown in Figures 3.2, 3.3 and 3.4, respectively. Placebo HFIM experiments for the three wild-type strains revealed that a very small proportion of pre-existing natural mutants were present. However, the relative proportion of the pre-existing resistant sub-population to the total population remained unchanged over time. Placebo did not exert any selective pressure on the heterogeneous bacterial populations; therefore no resistance amplification was observed (Figures 3.2 A, 3.3 A and 3.4 A).

On the other hand, in the presence of a selective pressure exerted by a sub-optimal dose of 30 mg once daily (AUC/MIC = 47), amplification of resistant mutants was observed for ATCC 25922 and EC28044. As shown in Figures 3.2 B and 3.4 B, a considerable reduction in total bacterial burden was observed at 4 h for both strains. This reduction was likely due to the rapid and preferential killing of the susceptible sub-population. However, the bacterial populations could not be controlled after 24 h despite repeated dosing, as the dose exposure was insufficient to hold the resistant sub-population from proliferating. For MG1655 (Figure 3.3 B), regrowth was apparent at 48 h with a sub-optimal dose of 80 mg once daily (AUC/MIC = 117). In all cases, resistant sub-population almost completely replaced the susceptible population over time. Further increasing the dose to 80 mg (AUC/MIC = 117) for ATCC 25922 (Figure 3.2 C) and EC28044 (Figure 3.4 C) resulted in suppression of resistance. Similarly, a dose of 120

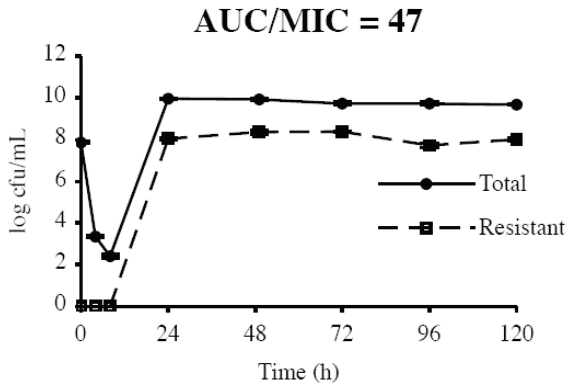
mg (AUC/MIC = 180) for MG1655 (Figure 3.3 C) led to resistance suppression. The clinical dose of moxifloxacin 400 mg (equivalent to AUC/MIC = 627) was sufficient to suppress the resistance development in all the three wild-type strains of *E. coli* (data not shown). A summary of the microbial responses observed with various pharmacodynamic exposures is depicted in Table 3.1.

Figure 3.2: Placebo response of ATCC 25922 (A). Response of ATCC 25922 with simulated dose of moxifloxacin. Regrowth observed at AUC/MIC ratio of 47 (B) and suppression observed with AUC/MIC ratio of 117 (C). Data as mean ± SD.

(A)



(B)



(C)

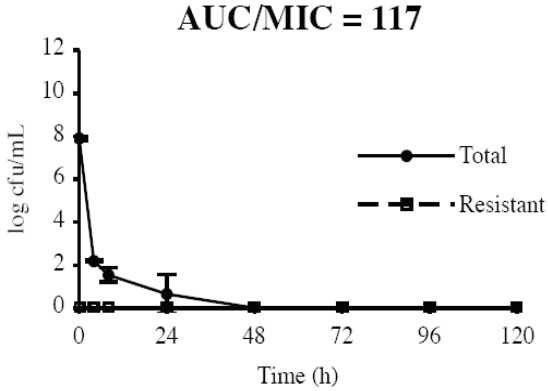
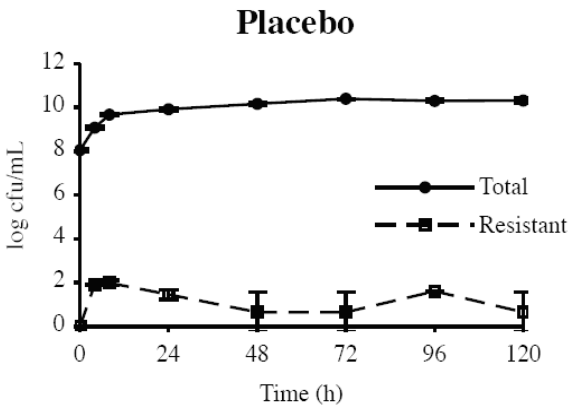
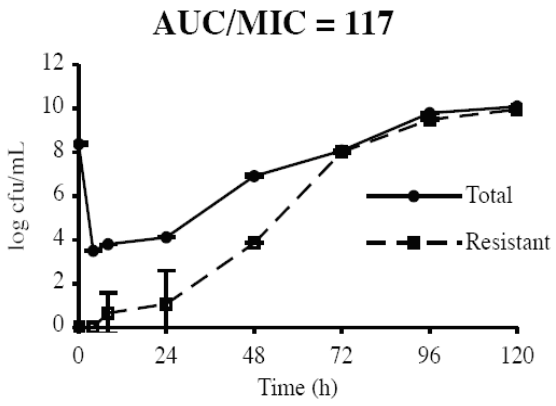


Figure 3.3: Placebo response of MG1655 (A). Response of MG1655 with simulated dose of moxifloxacin. Regrowth observed at AUC/MIC ratio of 117 (B) and suppression observed with AUC/MIC ratio of 180 (C). Data as mean ± SD.

(A)



(B)



(C)

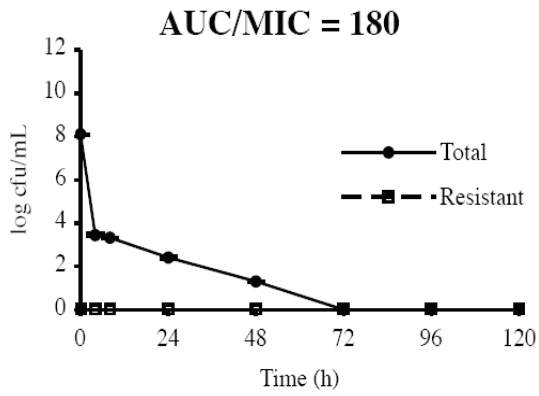
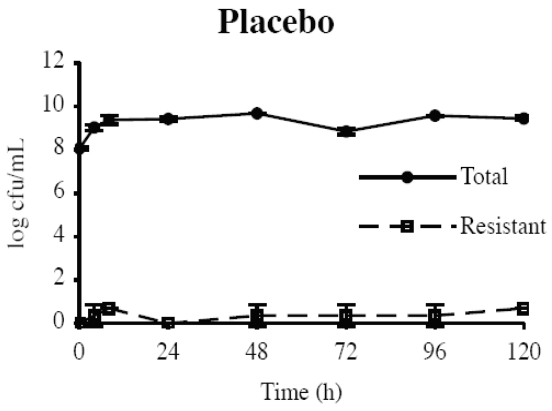
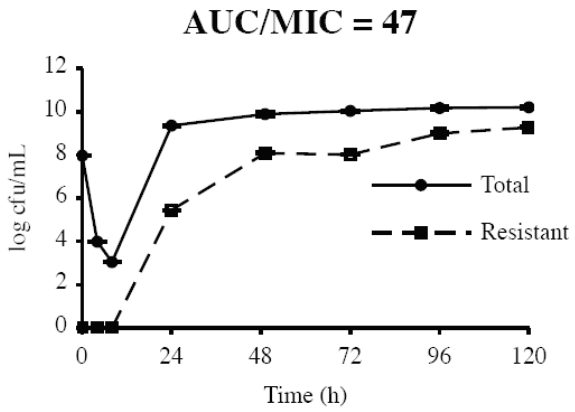


Figure 3.4: Placebo response of EC28044 (A). Response of EC28044 with simulated dose of moxifloxacin. Regrowth observed at AUC/MIC ratio of 47 (B) and suppression observed with AUC/MIC ratio of 117 (C). Data as mean ± SD.

(A)



(B)



(C)

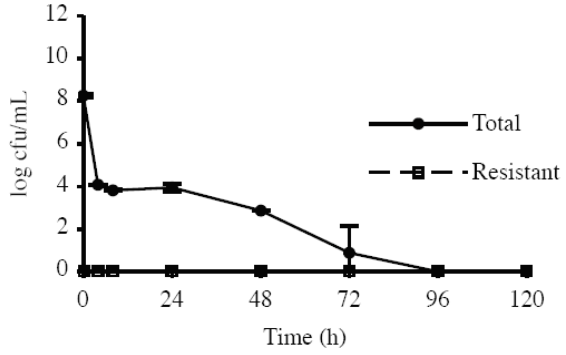


Table 3.1: Summary of bacterial responses observed with the simulated exposures of moxifloxacin.

AUC/MIC	47	117	145	180	627
Approximately equivalent dose	30 mg	80 mg	100 mg	120 mg	400 mg
ATCC 25922	Regrowth	Suppression	Suppression		
MG1655		Regrowth		Suppression	Suppression
EC28044	Regrowth	Suppression			Suppression

3.4.4 Confirmation of moxifloxacin resistance mechanisms

Isolates recovered from moxifloxacin-supplemented plates were sub-cultured at least three times on blood agar plates to confirm the resistant phenotype was stable. Susceptibility studies performed on these isolates revealed resistance to moxifloxacin with 8- to 32-fold increase in MIC (data not shown). Cross-resistance was also observed with ciprofloxacin and levofloxacin. Sequencing the QRDR of *gyrA* and *parC* revealed that all the resistant isolates had one amino acid substitution in *gyrA*. While all the resistant isolates derived from MG1655 had a S83L substitution, there was a D87G substitution in resistant isolates derived from ATCC 25922 and EC28044.

3.5 DISCUSSION

Enterobacteriaceae especially *E. coli* remains one of the major pathogens implicated in intra-abdominal infections (Rossi et al., 2006). These infections often harbor a high inoculum of bacteria and resistance is likely to develop subsequent to sub-optimal dosing. Hence, the knowledge of pharmacodynamics of antibacterial agents at a high inoculum is indispensable in order to formulate treatment strategies to suppress resistance.

Recently, emphasis has been placed on understanding pharmacodynamics of antimicrobial agents (especially fluoroquinolones) to improve clinical outcomes. Studies

by Forrest et al. and Drusano et al. were pioneering in this regard (Forrest et al., 1993; Drusano et al., 2004). In a retrospective analysis using patients receiving ciprofloxacin for nosocomial infections, Forrest and colleagues characterised the drug exposure determinants of treatment outcome. They demonstrated that the probability of a good clinical outcome was related to the AUC/MIC ratio. Their data suggested that the clinical and bacteriological outcomes could be significantly improved by optimizing the dosing exposure. In another study, Drusano et al. investigated the factors affecting the likelihood of a good microbiological or clinical outcome in patients with nosocomial pneumonia (2004). They advocated that only the age of patients and the achievement of an AUC/MIC ratio of ≥ 87 had a significant impact on pathogen eradication. Differences in clinical outcomes with age were probably related to underlying co-morbidities and the impact of the immune system in elderly individuals.

Understanding fluoroquinolone pharmacodynamics has also been targeted towards suppression of resistance. In cases where resistance is due to selective amplification of pre-existing resistant mutants, the knowledge of pharmacodynamics can be utilized to suppress resistance development by optimizing the drug exposure. Different pathogens and various experimental setups have been used to identify the drug exposure with highest probability of achieving this goal (Tam et al., 2007; Gumbo et al., 2004; Tam et al., 2005; Jumbe et al., 2003; Hansen et al., 2006). Using a non-neutropenic mouse model to examine levofloxacin exposure and resistance development in *Pseudomonas*

aeruginosa, Jumbe et al. put forward a mathematical model to describe the target exposure required to suppress the emergence of resistant bacterial sub-population (Jumbe et al., 2003). This model was extended and validated in two separate *in vitro* studies by Tam et al., where they investigated the impact of garenoxacin exposure intensity on resistance emergence in *Staphylococcus aureus* and *P. aeruginosa* (Tam et al., 2007; Tam et al., 2005). The authors suggested an optimal drug exposure could be used in suppressing resistance in *S. aureus* and *P. aeruginosa*. Other pathogens like *Mycobacterium tuberculosis* have also been examined for the propensity of *in vitro* resistance suppression (Gumbo et al., 2004). A second approach to suppress resistance has been put forward by Drlica et al., which is based on the concept that mutations in QRDR are likely to be sequential instead of simultaneous (Hansen et al., 2006). Thus a drug concentration (mutant prevention concentration) that prevents the amplification of first step mutation would also prevent subsequent mutations. The concept has evolved into minimizing the time in which the drug concentration is in the range that selectively enriches the resistant sub-population (mutant selection window).

Moxifloxacin pharmacodynamics has been investigated by many authors, but mostly in Gram-positive microorganisms like *S. aureus*, *Streptococcus pneumonia* and *Streptococcus pyogenes* (Firsov et al., 2000; LaPlante et al., 2007; MacGowan et al., 2003; Odenholt et al., 2002; Rodriguez-Cerrato et al., 2001; Hermsen et al., 2005). Anaerobes and *M. tuberculosis* have also been examined in this context (Noel et al.,

2005; Gumbo et al., 2004). In Gram-negative microorganisms, the pharmacodynamics of moxifloxacin have been investigated against *P. aeruginosa* and *E. coli* (MacGowan et al, 2003; Rodriguez-Cerrato et al., 2001; Hermsen et al., 2005; Odenholt et al., 2006). In particular, an *in vitro* study by Hermsen et al. compared the activity of levofloxacin plus metronidazole versus moxifloxacin in a mixed-infection model of *E. coli* and *Bacteroides fragilis* (Hermsen et al., 2005). Only one clinical isolate of *E. coli and B. fragilis,* and one dose exposure was studied for 24 h. Although this study aimed to compare combination versus monotherapy for intra-abdominal infections, the inoculum used in the study was 10^6 CFU/ml, which might not be reflective of the bacterial burden encountered clinically. The study also did not delineate the drug exposure necessary for resistance suppression.

Another study by Odenholt et al. compared the pharmacodynamics of moxifloxacin and levofloxacin against *S. pneumoniae*, *S. aureus*, *Klebsiella pneumoniae* and *E. coli* isolates with differing susceptibility in an *in vitro* kinetic model (Odenholt et al., 2006). The authors suggested that a maximal antibacterial effect was observed when AUC/MIC exceeded 100. However, a low inoculum of 5×10^5 CFU/ml and a 24 h time course was studied, which may not closely represent the pathophysiology of intra-abdominal infections and clinical treatment. As such, the pharmacodynamics of moxifloxacin to suppress resistance in *E. coli* has not been thoroughly studied.

Results from this study provide additional insights to our understanding of pharmacodynamics of moxifloxacin. First, in terms of the drug exposure necessary to counter-select resistance, three wild-type strains from different background were used to impart greater generalizability to our hypothesis. Our data suggested an AUC/MIC ratio between 117–180 would be needed. These results are in general agreement with Odenholt et al. and the difference could be due to the variations in strains examined. Second, *hollow* fiber system provided flexibility to test our hypothesis. This *in vitro* system enabled us to study high inocula of bacteria, which might be difficult in animal models since such a high inoculum could be associated with unacceptable mortality. It also allowed us to investigate a longer drug exposure duration, which has not been commonly reported in other infection models. Third, our observation of one *gyrA* mutation in all resistant isolates was consistent with findings of Morgan-Linnell et al. However, unlike their observation of an additional *parC* mutation in 85% of isolates, we found no *parC* mutation in any of the resistant isolates (Morgan-Linnell et al., 2009). One of the reasons for having these parC mutations could be attributed to the fact that most of the isolates had several-fold higher MIC than the randomly selected resistant isolates in this study. A higher selection threshold (e.g., 8-12x MIC) might be needed to detect these high-level resistant mutants reliably. A second reason could be that moxifloxacin possesses greater ability to prevent resistance development, which not extensively used in the previous study (Morgan-Linnell et al., 2009).

It should be noted that the results of this study are conservative, as the *in vitro* infection system lacked an immune response and represented only the direct relationship between drug exposure and microorganism. However, this allowed us to delineate an unambiguous pharmacodynamic response for testing our hypothesis. Resistance to fluoroquinolones could be a consequence of one or combination of multiple mechanisms (e.g., mutations in QRDR, efflux overexpression or mediated via *qnrA*). Only QRDR of *gyrA* and *parC* were screened for mutation as these are the primary targets (Morgan-Linnell et al., 2009) and mutations in *gyrB* or *parE* do not consistently contribute to significant increase in MIC (Morgan-Linnell et al., 2009). However, the possibility of *gyrB* or *parE* mutation(s) and efflux pump involvement in conferring resistance cannot be completely ruled out. This study was not designed to delineate the role of plasmid-mediated quinolone resistance.

In summary, our data suggest that a sub-optimal dose exposure of moxifloxacin may facilitate resistance amplification in a high inoculum of *E. coli*. The clinical dose of moxifloxacin of 400 mg was likely adequate in suppressing the emergence of resistance. The clinical relevance of this study needs further *in vivo* investigations.

CHAPTER 4

Impact of *recA* on Levofloxacin Exposure-Related Resistance Development

This chapter has been published: Singh et al., 2010

4.1 ABSTRACT

Genetic mutations are one of the major mechanisms by which bacteria acquire drug resistance. One of the known mechanisms to induce mutations is the SOS response system. We investigated the effect of disrupting *recA* - an inducer of SOS response, on resistance development using an *in vitro* HFIM. A clinical *Staphylococcus aureus* isolate and a laboratory wild-type strain of *Escherichia coli* were compared to their respective *recA*-deleted isogenic daughter isolates. Approximately 2×10^5 CFU/ml of bacteria were subjected to escalating levofloxacin exposures for up to 120h. Serial samples were obtained to ascertain simulated drug exposures, total and resistant bacterial burden. Quinolone resistance determining regions of *gyrA* and *grlA* (*parC* for *E. coli*) in levofloxacin-resistant isolates were sequenced to confirm the mechanism of resistance. Pre-exposure MIC of the *recA*-deleted isolates was 4-fold lower than their respective parents. In *S. aureus*, a lower AUC/MIC was required to suppress resistance development in the *recA*-deleted mutant (AUC/MIC > 23 versus AUC/MIC > 32 were necessary in the mutant and parent isolate, respectively), and prominent difference in

total bacterial burden was observed at 72h. Using an AUC/MIC of approximately 30, *E. coli* resistance emergence was delayed by 24h in the *recA*-deleted mutant. Diverse mutations in *gyrA* were found in levofloxacin-resistant isolates recovered. Disruption of *recA* provided additional benefits apart from MIC reduction, attesting to its potential role for pharmacologic intervention. Clinical relevance of our findings warrants further investigations.

Keywords: SOS response, *recA*, levofloxacin, *Escherichia coli*

4.2 INTRODUCTION

Resistance to antibacterial agents is a major health concern that is widely spreading. Increasingly, common drugs like β-lactams and fluoroquinolones have been rendered ineffective in the clinic. Consequently, it is necessary that new therapeutic interventions are developed to combat resistance and meet the medical demands of treating infections due to pathogens resistant to available antibacterial agents.

Resistance to antibacterial agents occurs frequently by genetic mutations or the acquisition of genetic material carrying resistance determinants. Evidence suggests that genetic mutations are one of the main mechanisms of defense employed by bacteria against many antibacterial agents (Almahmound et al., 2009; Chen et al., 2001). Bacteria can escape the lethal effect of antimicrobial agents by acquiring mutations in target binding site, genes regulating efflux pumps and genes encoding porin synthesis (Chuanchuen et al., 2008; Morgan-Linnell et al., 2009; Oteo et al., 2008; Von Groll et al., 2009). Needless to say, mutations are important for acquiring resistance and finding ways to reduce mutation rates in bacteria might help us to combat resistance.

Bacteria commonly develop spontaneous mutations as a consequence of error prone DNA replication. Some of these random mutations may confer resistance to antibiotics. Lately, studies have suggested that bacteria also play an active role in inducing mutations in the event of exposure to certain class of antibiotics or other environmental stress (Cirz

et al., 2005; Riesenfeld et al., 1997). One of the mechanisms by which these mutations are induced is the SOS response system (Erill et al., 2007). SOS response system is a global response to DNA damage in which DNA repair and mutagenesis are induced. A central part of the SOS response is the depression of various SOS genes, which are under the direct and indirect transcription control of a repressor - LexA. Another important protein involved in regulating the SOS response is RecA (the sensor of the system). RecA is a recombinase protein that has coprotease activity, functions in DNA strand exchange and facilitates replicative bypass of DNA lesions. In a non-triggered state, LexA binds as a dimer in the promoter region of SOS genes and down regulates its own expression and that of other SOS genes. Formation of single-stranded DNA or a stalled replication fork resulting from DNA damage acts as a trigger for the SOS system and activates RecA. Upon activation, coprotease activity of RecA causes autocatalytic cleavage of the LexA dimer and leads to derepression of the SOS genes. The LexA regulon, in most cases, includes recombination and repair genes - *recA*, *recN*, and *ruvAB*, nucleotide excision repair genes - *uvrAB* and *uvrD*, the error-prone DNA polymerase (pol) genes - *dinB* (encoding pol IV) and *umuDC* (encoding pol V) and DNA polymerase II, along with other genes. Thus, induction of the SOS response trades long term fidelity with short term viability by mediating processes such as cell division inhibition, excision repair, up-regulation of tri-carboxylic acid cycle and error prone replication. Overall, this process leads to a higher rate of mutant formation.

Since spontaneous mutations are an integral part of the bacterial adaptation and do not provide obvious means to target, the pathways that lead to mutations linked with antibiotic resistance (such as the SOS response) seems to be a logical and potentially lucrative target for pharmacological intervention. Hence, we attempted to investigate the effect of RecA (one of the key regulatory proteins involved in SOS response and a potential pharmacologic target) on resistance development. Two clinically important pathogens - *Staphylococcus aureus* and *E. coli* were selected as our test microorganisms. *S. aureus* is a Gram-positive bacterium that is known to cause pneumonia and skin infections. *E. coli* is a Gram-negative bacterium that is a common cause of intra-abdominal and urinary tract infections.

4.3 MATERIALS AND METHODS

4.3.1 Antimicrobial agent

Levofloxacin hydrochloride was purchased from Waterstone Technologies (Carmel, IN). A stock solution was prepared by dissolving the powder in water and stored in aliquots at -70 °C. Prior to each investigation, an aliquot of the stock solution was thawed and diluted accordingly with cation-adjusted Mueller-Hinton broth (Ca-MHB) (BBL, Sparks, MD) or sterile water.

4.3.2 Microorganisms

The community-associated methicillin-resistant *S. aureus* isolate (ASAU021) that was used in the study was obtained from the Network on Antimicrobial Resistance in *Staphylococcus aureus* (strain NRS384). Previous molecular investigations revealed that this isolate was positive for *mecA* and belonging to the USA300 genotype (McDougal et al., 2003). PCR analysis (detailed below) revealed a point mutation in *grlA* (S80F). Using a temperature sensitive construct (pMAD), a *recA*-deleted isogenic daughter isolate (ASAU022) was derived (Cirz et al., 2007). A *recA*-deleted derivative (MG1655Δ*recA*) was also obtained from a wild-type *E. coli* (MG1655) strain. The bacteria were stored at -70 ^{o}C in Protect storage vials (Key Scientific Products, Round Rock, TX). Fresh isolates were sub-cultured twice on 5% blood agar plates (Hardy Diagnostics, Santa Maria, CA) and incubated for 24 h at 35 °C prior to each experiment.

4.3.3 Susceptibility studies

Levofloxacin MIC was determined by a modified broth macrodilution method as described by the Clinical and Laboratory Standards Institute (CLSI, 2007). The final bacteria concentration in each macrodilution tube was approximately 5 x 10^5 CFU/ml of Ca-MHB. Serial two-fold dilutions of levofloxacin were used. The MIC was defined as the lowest concentration of drug that resulted in no visible growth after 24 h of

incubation at 35 °C in ambient air. The studies were conducted in triplicate and were repeated at least once on separate days.

4.3.4 Time-kill studies

To elucidate the impact of *recA* deletion on bacterial response to static levofloxacin exposures, time-kill studies were conducted with escalating concentrations of levofloxacin (placebo control, 0.5x MIC and 2x MIC). On the day of the experiment, an overnight culture of each isolate was inoculated into pre-warmed Ca-MHB and incubated further at 35 °C until log-phase growth. The bacterial suspension was diluted with Ca-MHB based on absorbance at 630 nm; so that each flask had 16 ml of bacterial suspension at approximately 2x 10^5 CFU/ml. The experiments were conducted in a shaking water bath set at 35 °C. Serial samples (500 µl) were obtained over 24 h (baseline, 1, 2, 4, 8, 12, and 24 h) from each flask in duplicate and the viable bacterial population was determined by quantitative culture. Before plating, the bacterial samples were centrifuged at 10,000x *g* for 15 minutes at 4 ^{o}C and reconstituted with sterile normal saline to their original volumes in order to minimize drug carryover effect. Total bacterial populations were quantified by spirally plating (Spiral Biotech, Bethesda, MD) serial 10x dilutions of the samples on Mueller-Hinton Agar (MHA) plates (BD Diagnostics, Sparks, MD). The MHA plates were incubated in a humidified incubator

(35 °C) for up to 24 h, and the bacterial density was quantified by visual enumeration of the colonies. The theoretical lower limit of detection was 400 CFU/ml.

4.3.5 Hollow fiber infection model studies

To further elucidate the impact of *recA* deletion on resistance development under more clinically relevant drug exposures, an *in vitro* HFIM was used. The schematics of the infection model have been described in detail previously (Tam et al., 2007). The inocula (20 ml) were prepared as described above and a final inoculum of approximately 2x 10^5 CFU/ml was used for each isolate. Based on the mutation frequency of the bacteria (approximately 1 in 10^{7-8}), the starting inocula were deemed to be homogenous and pre-existing mutants were not anticipated at baseline. The isogenic pair of *S. aureus* isolates (ASAU021 and ASAU022) was subjected to escalating levofloxacin exposures (AUC/MIC ratio ranging from 0 - 70, adjusted for their respective MIC) for up to 5 days; repeated doses were given once every 24 h with a targeted elimination half-life of 5 - 7 h (Pea et al., 2003). To provide some generalizability of the results, an isogenic pair of *E. coli* isolates (MG1655 and MG1655Δ*recA*) was also exposed to one target (the most informative) AUC/MIC, based on the results from experiments conducted on *S. aureus*. To ascertain the pharmacokinetic profiles simulated in the infection models, serial samples were obtained on alternate days from the circulating loop of the system. Levofloxacin concentration in these samples was assayed by a validated method outlined

below and a one compartment linear model was fit to the observed concentration-time profiles using the ADAPT II program (D'Argenio et al., 1997). In addition, serial samples were also obtained daily in duplicate (baseline, 4, 8, 24, 28, 48, 52, 72, 96, 120 h, pre-dose when applicable) from the infection models to determine viable bacterial burden over time. The samples were washed once in sterile saline and diluted 10x serially, before plating (50 µl) on drug-free MHA and MHA supplemented with levofloxacin (at 3x and 12x MIC). Levofloxacin-supplemented plates were made in two concentrations to detect isolates with different magnitude of reduced susceptibility (drug resistance). The generally accepted interday deviation for MIC testing is a two-fold difference in MIC value, hence a 3x MIC supplemented plate would allow reliable detection of population with reduced susceptibility. In addition, 12x MIC levofloxacin-supplemented plates were used to further delineate the mechanism of resistance. The MHA plates were incubated at 35 °C in humidified ambient air for up to 72 h before the colony-forming units were enumerated visually.

4.3.6 Analysis of drug concentration

Samples were analyzed using an HPLC system consisting of a Shimadzu SIL-HTC autosampler, degasser and binary pumps. Separation was performed using Zorbax SB-C3 5 µm, 50 x 2.1 mm. The mobile phase was delivered at 0.7 ml/min as a 1.5 minutes linear gradient from 95A : 5B to 2A : 98B; where A was water with 0.5% formic acid and

B was acetonitrile with 0.5% formic acid. The mass spectrometer (API3000) was operated in ES positive ionization mode. Data were acquired using Q1 mass at 362.08 and Q3 mass at 318.1. The samples (50 µl) were mixed with 10 µL of internal standard (propranolol 15 µg/ml in water), cleaned up by addition of 300 µL of methanol followed by vortexing and centrifugation. The supernatant (300 µl) was diluted with 200 µl water. Levofloxacin standards (5 µl of stock solution added to 45 µL of Ca-MHB) were prepared similarly. The assay was linear in the concentration range from 0.001 to 5 mg/l.

4.3.7 Confirmation of resistance

Two random isolates from levofloxacin-supplemented plates (at each concentration 3x MIC and 12x MIC) were recovered at the end of each experiment which regrowth was observed. Susceptibility testing was repeated to ascertain levofloxacin resistance. The mechanism of levofloxacin resistance was elucidated by PCR of the quinolone resistance determining regions (QRDRs) of *gyrA* and *grlA* (or *parC* for *E. coli*) genes. For *S. aureus*, the thermocycling conditions consisted of an initial denaturing step of 94 °C for 15 minutes, followed by 35 cycles of 94 °C for 1 minute, 51 °C for 1 minute and 72 °C for 1 minute, and a final extension at 72 °C for 10 minutes. Primer sequences used are as detailed in Table B of the Appendix. For *E. coli*, the thermocycling conditions and the primers used have been detailed previously (Singh et al., 2009).

4.4 RESULTS

4.4.1 Susceptibilities

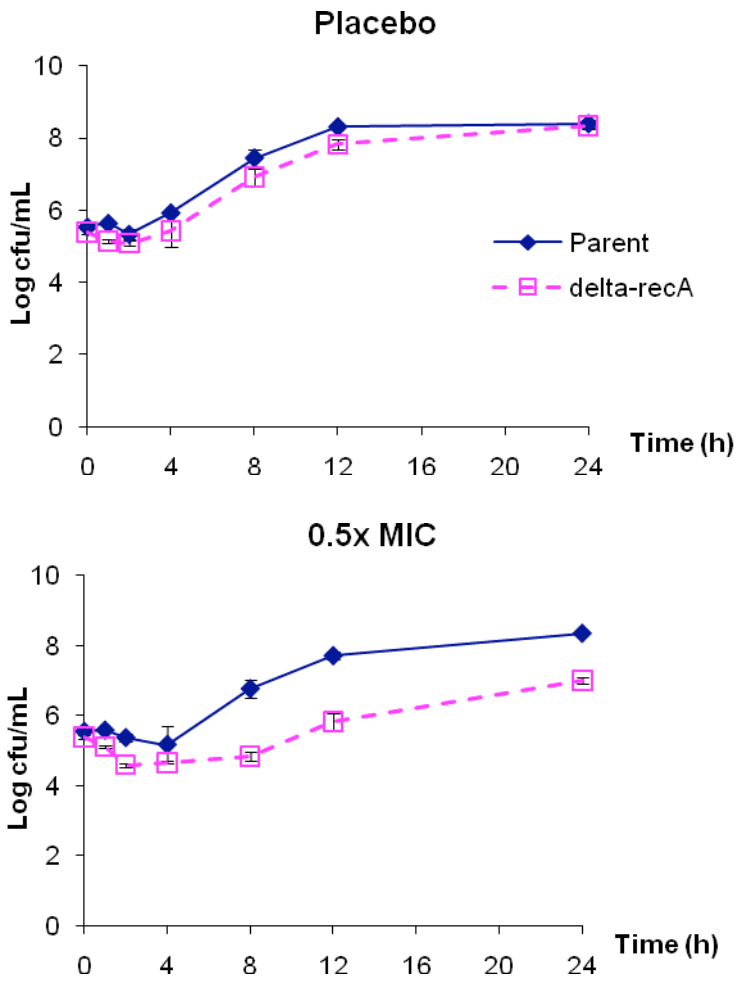
The MIC of ASAU021 and ASAU022 to levofloxacin were found to be 1 mg/l and 0.25 mg/l, respectively. In addition, the levofloxacin MICs for MG1655 and MG1655Δ*recA* were 0.016 mg/l and 0.004 mg/l, respectively. Generally speaking, the deletion of *recA* in a variety of bacteria conferred a reduction in levofloxacin MIC by 2- to 8-fold (data not shown).

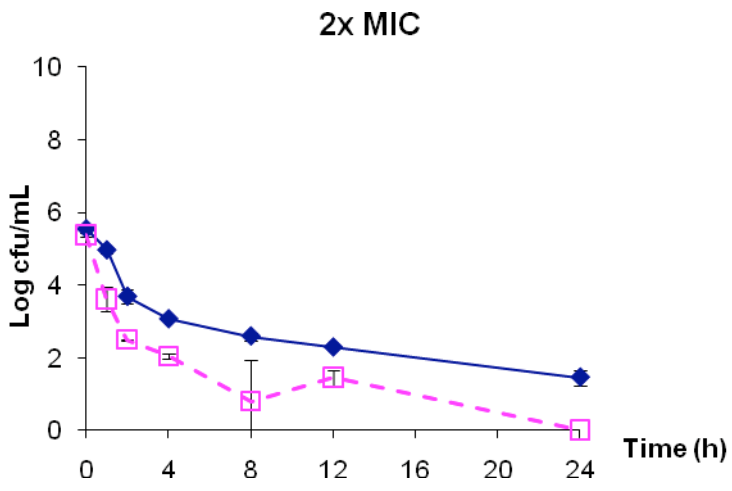
4.4.2 Time-kill studies

The responses observed for the two isogenic strains of *S. aureus* and *E. coli* using static levofloxacin concentrations are shown in Figure 4.1. For each pair of isolates, a comparison was made after normalizing for their respective MIC to determine if there was an additional benefit from the *recA* deletion beyond decrease in MIC. Deletion of *recA* did not appear to change the growth rates considerably in either pair in the placebo control experiments. The bacterial burden observed for the parent isolates were either identical or higher than the *recA*-deleted mutants at all times. The *recA*-deleted *S. aureus* isolate showed a slower re-growth rate (at 0.5x MIC) and a steeper kill rate (at 2x MIC), compared to the parent isolate. In contrast, the *recA*-deleted *E. coli* isolate only demonstrated a marginally slower re-growth rate; the difference (if any) between the isogenic isolates was much less pronounced.

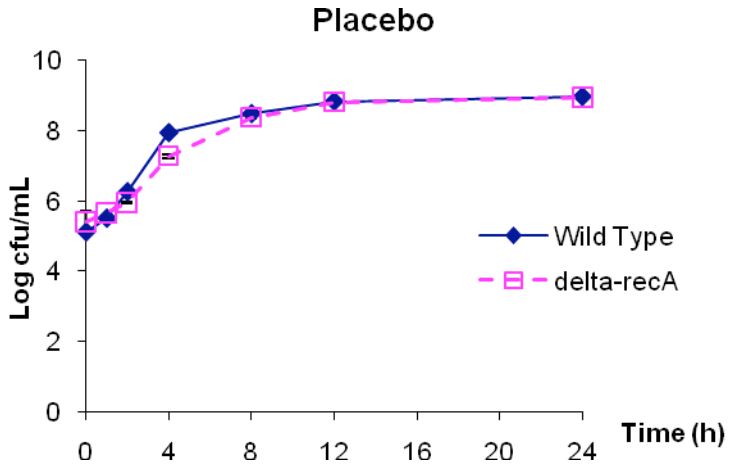
Figure 4.1: Bacterial responses in time-kill studies: *S. aureus* (A); *E. coli* (B)

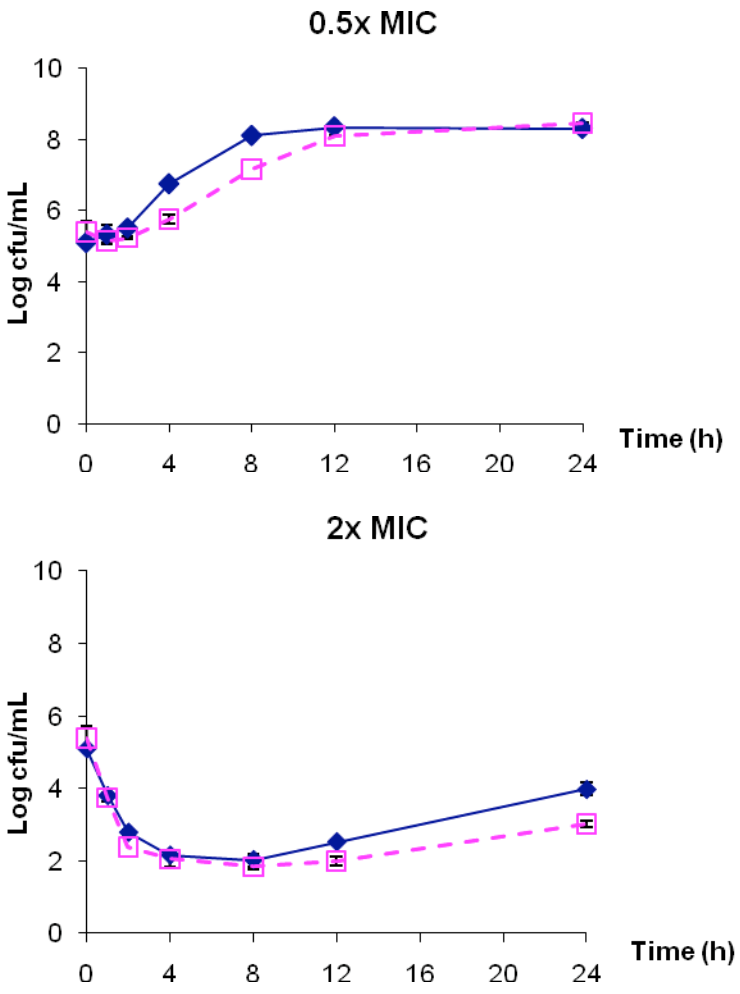
A




2x MIC
Log cfu/mL
10
8
6
4
2
0
0
4
8
12
16
20
24
Time (h)

B


Placebo
Log cfu/mL
10
8
6
4
2
0
0
4
8
12
16
20
24
Time (h)
Wild Type
delta-recA



Data shown as mean ± standard deviation

4.4.3 Hollow fiber infection model studies

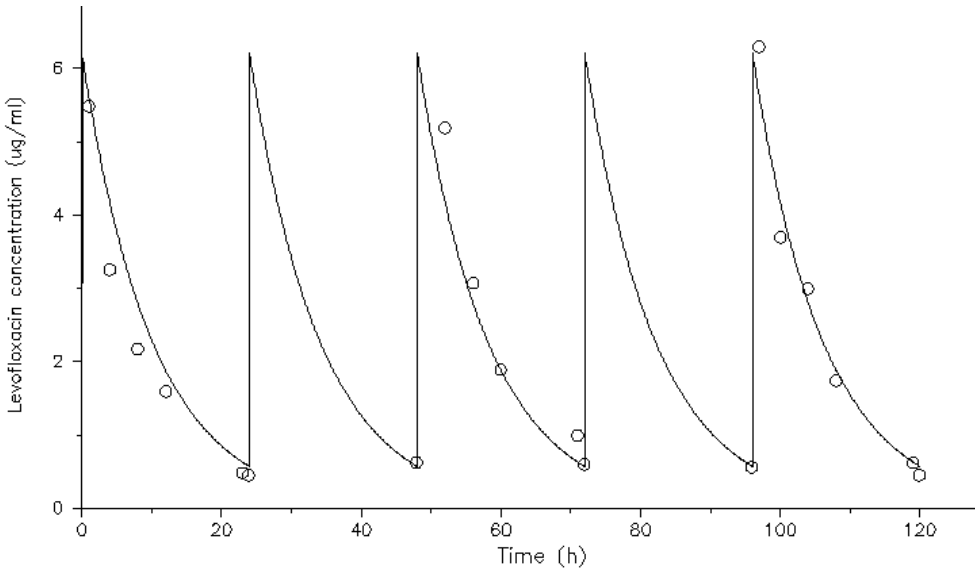
Simulated drug exposures in the infection models were satisfactory; a typical pharmacokinetic profile observed is shown in Figure 4.2. All the simulated drug exposures had $r^2 > 0.87$ and half-lives were within the target of 5 - 7 h. Bacterial (*S. aureus*) responses to selective levofloxacin AUC/MIC exposures are shown in Figure 4.3. Overall, placebo did not exert a selective pressure on the bacterial population; the total population was not suppressed but no resistance emergence was observed (data not shown). With low drug exposures, there was a significant reduction in bacterial burden initially, followed by regrowth and the emergence of resistance. Tracking the proportion of bacterial sub-populations with reduced susceptibilities to levofloxacin (with different levofloxacin-supplemented plates), it was demonstrated that the bacterial population was gradually replaced by mutants with reduced levofloxacin susceptibility over time. In contrast, sustained suppression of bacterial population was observed with elevated drug exposures.

To provide a direct assessment of the impact of RecA on resistance development, the experimental data were analyzed in 2 ways. To suppress resistance development over time, a levofloxacin AUC/MIC ratio greater than 32 was necessary for the *S. aureus* parent isolate. In contrast, an AUC/MIC ratio greater than 23 was required for the *recA*-deleted mutant; no resistance emergence was observed with an AUC/MIC ratio of 31. At this drug exposure, bacterial population eradication was observed for the *recA*-deleted

mutant, but not with the parent isolate. In another analysis, the total bacterial burden observed for the 2 isogenic *S. aureus* isolates after 72 h of drug exposure were compared, as shown in Figure 4.4. After adjusting for MIC, there was a 'left-shift' observed in the relationship between bacterial burden of the *recA*-deleted mutant and levofloxacin AUC/MIC. In both analyses, the most drastic difference between the isogenic pair appeared to be around AUC/MIC of 30. Consequently, an addition set of experiment (with similar design) was performed in an isogenic pair of *E. coli* using an AUC/MIC of 30 as the target.

The observed bacterial (*E. coli*) responses to a targeted levofloxacin AUC/MIC exposure are shown in Figure 4.5. Surprisingly, re-growth and resistance emergence were observed with both isolates under an almost identical pharmacodynamic exposure. Of note, there was a delay of approximately 24 h in the bacterial burden time course of the *recA*-deleted mutant, as shown in Figure 4.6. The less pronounced difference in bacterial response between the *E. coli* wild-type and the *recA*-deleted mutant was consistent with the results in time-kill studies (Figure 4.1B).

Figure 4.2: A typical pharmacokinetic profile observed in the HFIM.



Open circles depict experimental observations, solid line represents the best-fit model

Parameter estimates of the best-fit model:

$R^2 = 0.947$

Elimination half-life = 6.96 h

Clearance = 0.017 L/h

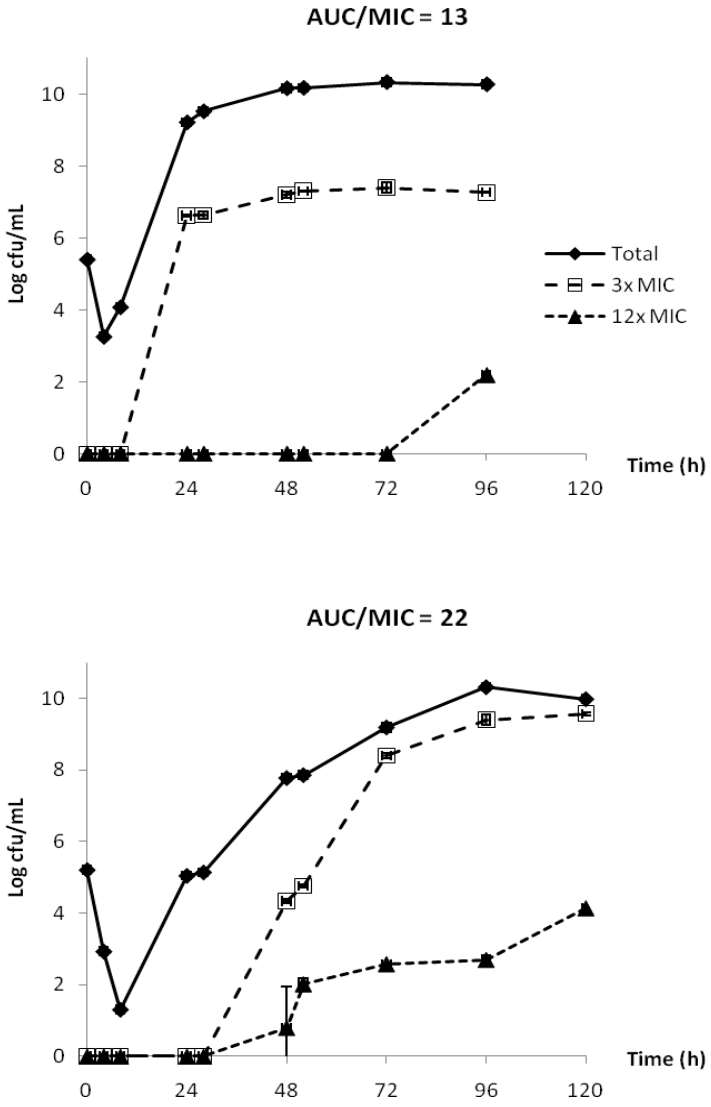
$AUC_{0\text{-}24}$ = 62.4 mg.h/L

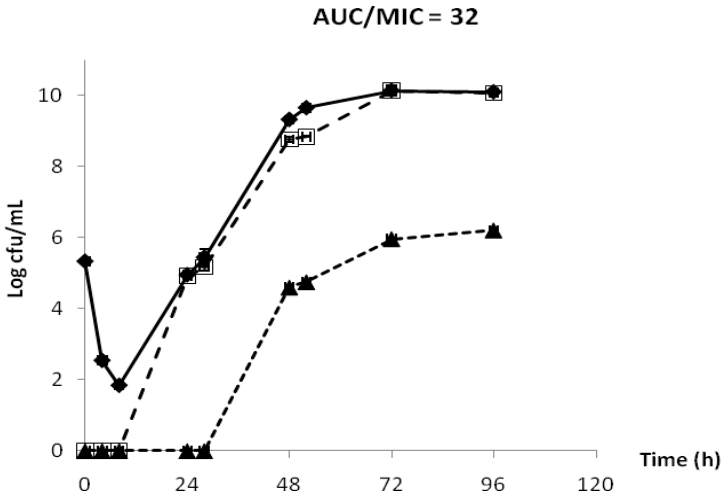
MIC = 1 mg/L (parent *S. aureus*)

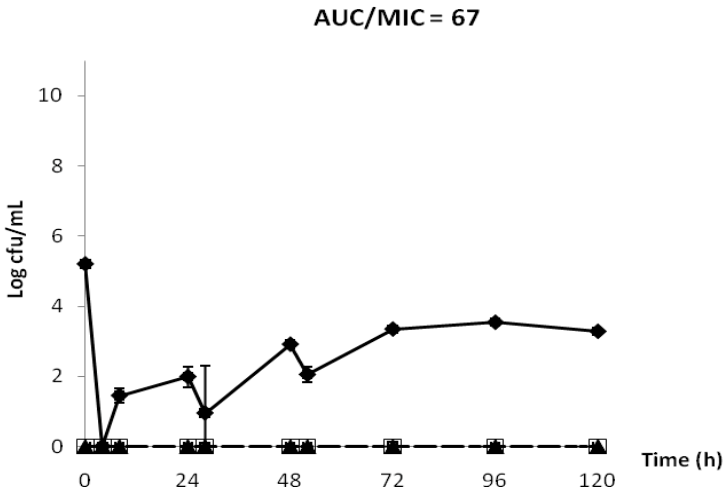
$AUC_{0\text{-}24}$ / MIC = 62.4

Figure 4.3: Observed bacterial responses to various levofloxacin exposures in HFIM: parent *S. aureus* ASAU021 (A); *recA* deleted mutant ASAU022 (B)

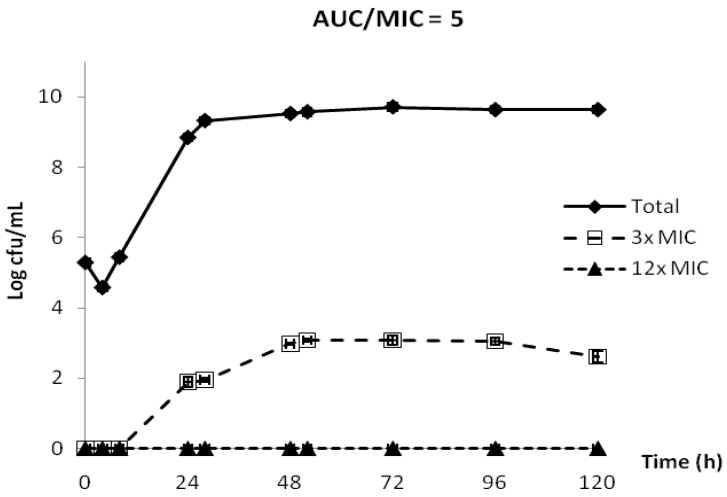
A

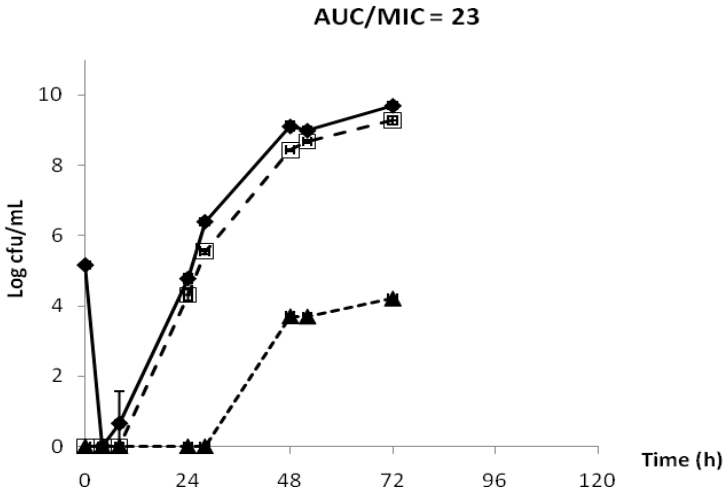


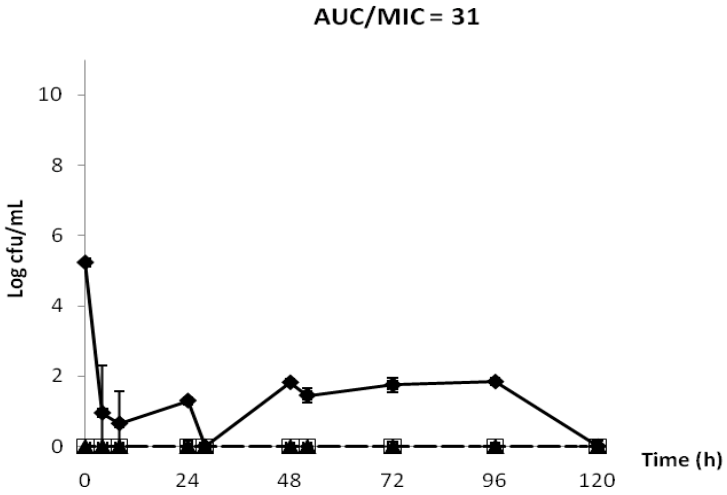

AUC/MIC = 32
Log cfu/mL
10
8
6
4
2
0
0
24
48
72
96
120
Time (h)

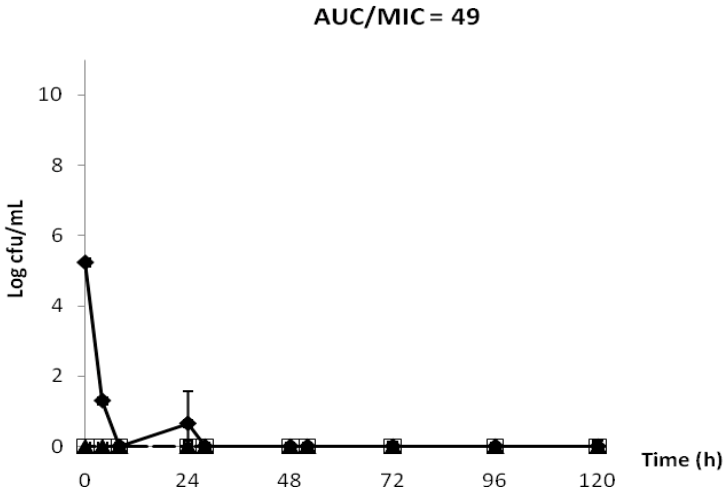

AUC/MIC = 67
Log cfu/mL
10
8
6
4
2
0
0
24
48
72
96
120
Time (h)

B


AUC/MIC = 5
Log cfu/mL
10
8
6
4
2
0
0
24
48
72
96
120
Time (h)
Total
3x MIC
12x MIC


AUC/MIC = 23
Log cfu/mL
10
8
6
4
2
0
0
24
48
72
96
120
Time (h)





Data presented as mean ± standard deviation. The experiments were performed up to 120 h or when the hollow fiber cartridge could no longer confine the bacteria, whichever occurred earlier.

Figure 4.4: Comparison of observed total bacterial burden for the parent and *recA*-deleted strains of *S. aureus* after 72 h of levofloxacin exposure

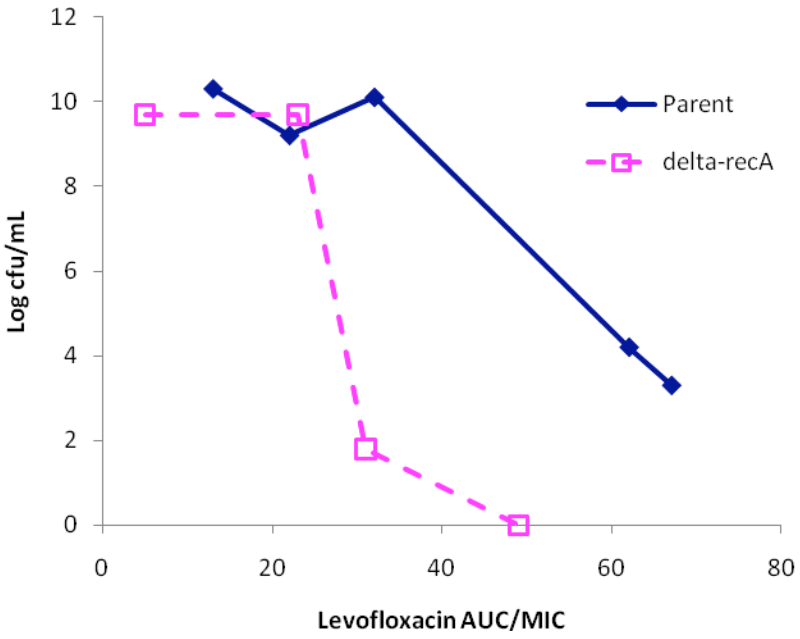
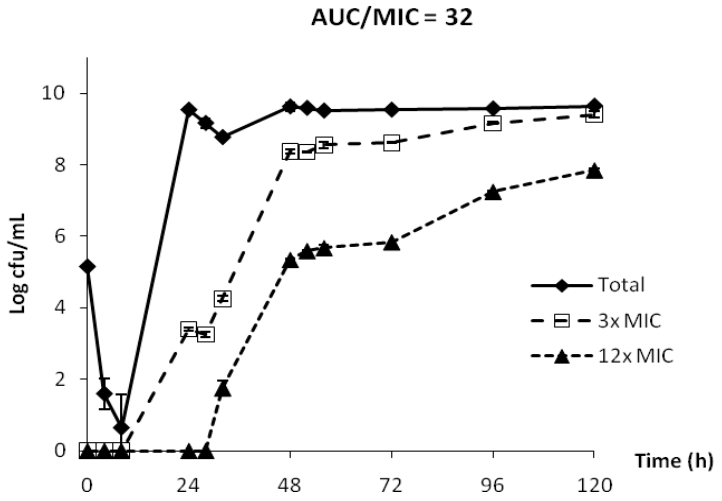
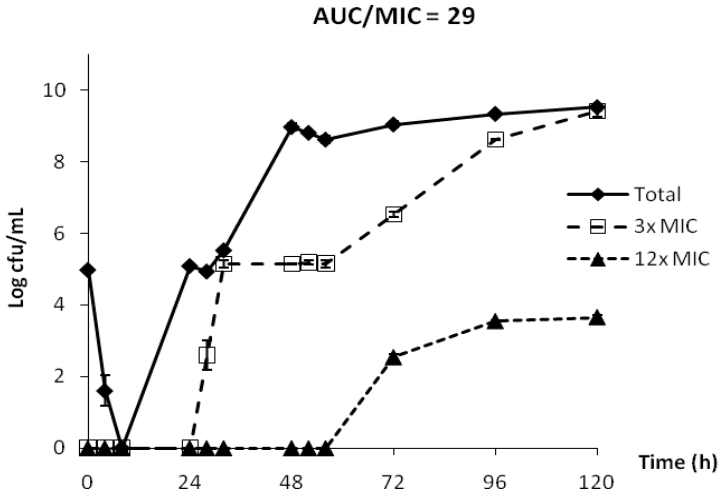


Figure 4.5: Observed bacterial responses to similar levofloxacin exposures: wild-type *E. coli* (A); *recA*-deleted *E. coli* mutant (B)

A

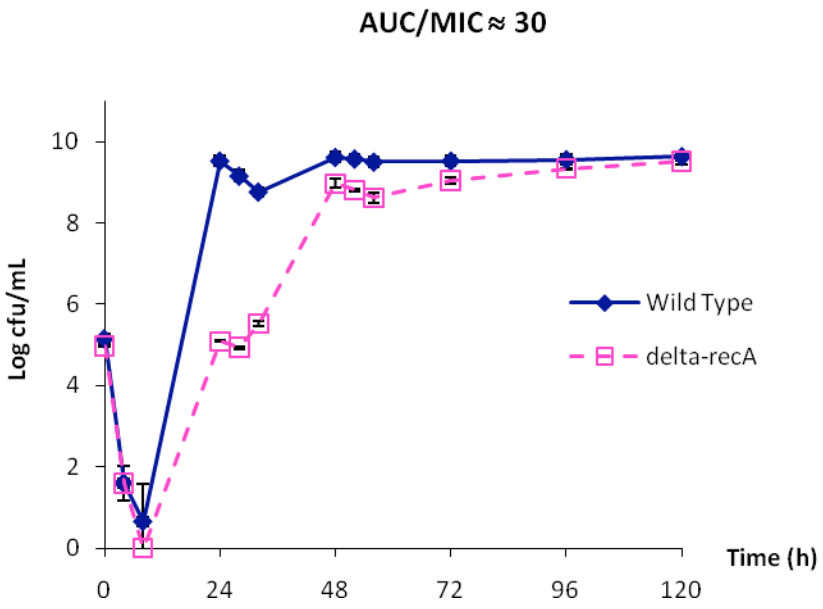


B



Data presented as mean ± standard deviation

Figure 4.6: Comparison of observed total bacterial burden of wild-type and *recA*-deleted *E. coli* to similar levofloxacin exposures in HFIM.



Data presented as mean ± standard deviation

4.4.4 Mechanisms of levofloxacin resistance

The parent *S. aureus* isolate (ASAU021) was found to have a point mutation (S80F) in *grlA*, despite exhibiting a susceptible phenotype. All *S. aureus* isolates recovered from levofloxacin-supplemented plates were found to have elevated levofloxacin MIC (4- to >48- fold increase) compared to their parent strain. Additional mutations in *gyrA* (e.g., S84A, S84L, S84P, E88G, and E88K) were found in all the levofloxacin-resistant isolates, but no one predominant point mutation was noted. Only one additional mutation was found in *grlA* (V82L); this resistant isolate was derived from the parent isolate exposed to levofloxacin AUC/MIC ratio of 32 - the most resistance-selecting drug exposure examined.

Similarly, *E. coli* isolates recovered from levofloxacin-supplemented plates were found to have elevated levofloxacin MIC (8- to 96-fold increases) compared to their parent strain. In contrast to *S. aureus*, only mutations in *gyrA* (e.g., S83L, D87G, and D87Y) were found. No mutation in the QRDR of *parC* was detected.

4.5 DISCUSSION

Mutations are one of the primary mechanisms of resistance to antibacterial agents. The SOS system is a stress response system which generates mutants by means of activating error prone DNA polymerases. The two key regulatory proteins of this pathway are LexA and RecA. Our study attempted to provide insights into SOS system as a potential target for therapeutic intervention to suppress emergence of resistance. To our knowledge this is the first study to examine RecA as a pharmacologic target using simulated human drug exposures.

Results from a previous study targeting the SOS response system to suppress resistance were promising. Cirz et al. studied the induction of mutation by fluoroquinolones and the impact of LexA cleavage on the emergence of resistance (2005). It was demonstrated that ciprofloxacin induced a 10^6-fold increase in mutational frequency in *E. coli*. It was further shown an *E. coli* strain having a non-cleavable LexA (thus unable to activate the SOS response) was less amenable to resistance emergence as compared to its parent wild-type strain in a neutropenic murine thigh infection model. While 3% of the wild-type population developed resistance, there was no resistant mutant observed in the *lexA*-deleted population, when a 0.5 mg/kg dose (approximately equal to AUC/MIC = 35) of ciprofloxacin was administered. Interestingly, this was the range of AUC/MIC (~30) where we observed the most effect of *recA* deletion in our study.

In this study we further extended the concept of inhibiting the SOS response system. We targeted the second regulatory protein (RecA) to investigate the effect of SOS inhibition on resistance emergence. In contrast to the study by Cirz et al., where only one dose was tested in the animal infection model, multiple simulated human drug exposures in an *in vitro* hollow fiber infection model were used. This experimental setup also allowed investigations for a longer duration of exposure and the direct effect of inhibition of SOS response without the potential interference of (residual) immune system.

Our study revealed RecA as a potential pharmacologic target from several aspects. Firstly, *recA* deletion itself resulted in a 4-fold reduction in levofloxacin MIC. Using the same dose of an antibacterial agent would result in a higher pharmacodynamic exposure (AUC/MIC ratio), if RecA were fully inhibited (pharmacologically or otherwise). For example, a clinical dose of levofloxacin (500 mg daily) would provide an AUC/MIC of approximately 40 for the clinical *S. aureus* isolate (which resistance emergence could occur over time), but the same dose of levofloxacin would have provided an AUC/MIC of approximately 160 when RecA is inhibited (which resistance development would be unlikely). Secondly, we also attempted to explore if there was any additional benefit of *recA* deletion after normalizing drug exposure to MIC. Our results suggested that *recA* deletion delayed the onset of resistance emergence in *E. coli* apart from conferring a reduction in MIC. Thirdly, we observed a marked difference between the *S. aureus*

parent and its *recA*-deleted isolate (Figure 4.4). As the AUC/MIC ratio exceeded 30 (a selective pressure of adequate intensity), there was a pronounced difference in total bacterial burden after 72 h. Keeping in mind this decrease in bacterial burden and delay in onset of resistance, a 'hit hard and early' approach could be applied to maximize the benefit of RecA inhibition. Finally, from the molecular aspect, since no pre-existing mutants were expected to be present at baseline, it was believed that isolates with diverse mutation patterns in QRDR arose as a result of the SOS response pathways. Resistance development was most favored and likely observed with a low to intermediate drug exposure. The resistance selection pattern in relation to pharmacodynamic exposure was consistent with the inverted-U phenomenon reported previously (Tam et al., 2007).

It was evident that mutations (via SOS response or otherwise) could not be completely obliterated with *recA* deletion. RecA is an important and early step in SOS response (Mesak et al., 2008), bacteria cannot rely on this primary rescue pathway when this gene is disrupted. However, other gene(s) may take over after some time delay. Possible reasons for this postulation could be redundancy in the pathways that regulate SOS response. It has been suggested that there are LexA and RecA independent pathways to trigger the SOS response (Perez-Capilla et al., 2005). For example, several β-lactams can induce translesion synthesis and mutagenesis by activating *dinB,* which is independent of the LexA / RecA regulatory system. In addition, fluoroquinolones have also been suggested to simulate intra- and inter-chromosomal recombination in *E. coli*

through mechanism that does not require LexA cleavage (Lopez et al., 2007). Results of our study indicated that in comparison to *S. aureus,* results in *E. coli* were less pronounced for the target AUC/MIC tested. However, a conclusive judgment cannot be made since only one dose exposure was tested. A wider range of experimental drug exposures would be necessary to confirm the true difference (if any).

It should be noted that our results could have been biased by imprecision of MIC determination. We used a geometric dilution series in drug concentration in the study, which means that a four-fold MIC difference between wild-type and *recA*-deleted isolates could actually be anywhere between 2- to 8-fold. The observed difference in bacterial response after normalizing to 'identical AUC/MIC' was relatively small (within 2-fold difference), and could have been entirely due to MIC variations. However, similar trends observed in two microorganisms were compelling enough to conclude that RecA inhibition was beneficial, as the probability of a systemic bias towards true lower MIC measurements is not anticipated to be high.

In conclusion, our study provided useful insights into a potential target to combat the looming danger of antibiotic resistance, and further investigations are warranted. As suggested earlier based on our results, one possible therapeutic strategy could be to hit hard and early with a fluoroquinolone when RecA is inhibited, before any secondary pathways to induce mutation could be activated. An interesting future study could be to

evaluate the impact of other SOS response mutants (including double mutants) in resistance emergence. Further *in vivo* experiments to investigate the impact of RecA inhibition in the presence of an intact immune system would also be valuable.

CHAPTER 5

Temporal Relationship of Two Main Mechanisms of Fluoroquinolone Resistance

5.1 ABSTRACT

Overexpressed efflux pumps and target site mutations are important mechanisms of fluoroquinolone resistance. The relationship and interplay between these mechanisms are not well understood. We examined the temporal appearance as well as the contribution of efflux pump (*acrAB*) overexpression and topoisomerase gene mutations to the emergence of levofloxacin resistance in *Escherichia coli*. A wild-type *E. coli* strain and its *acrAB*-deleted (Δ*acrAB*) isogenic derivative were studied. Intracellular accumulation was measured by liquid chromatography-tandem mass spectrometry (LCMS/MS) after levofloxacin (10 μg/ml) exposure for 20 min. Approximately 2x 10^6 CFU of the wild-type strain was exposed to sequentially escalating levofloxacin exposures over 10 days [area under the concentration-time curve over 24 h at steady state divided by the MIC (AUC/MIC) 7 to 3200] in an *in vitro* hollow fiber infection model (HFIM). Serial samples were obtained to validate simulated drug exposures and to quantify bacterial burden with different levofloxacin susceptibility. PCR and qRT-PCR were used to confirm the mechanism(s) of resistance. Additionally, both strains were exposed to a similar levofloxacin AUC/MIC. Levofloxacin MICs were 0.032 μg/ml for wild-type and

0.008 μg/ml for Δ*acrAB*. Intracellular levofloxacin concentration quantified in Δ*acrAB* was ~2x higher than wild-type. Resistant isolates recovered early (24 h) had 2-6x MIC elevation with *acrAB* overexpression (2-8x) but no point mutation. In contrast, high-level (≥ 64x MIC) resistant isolates with target site mutations (*gyrA* S83L +/- *parC* E84K) were selected more readily after 120 h; regression of *acrAB* overexpression was observed at 240 h. Early low-level levofloxacin resistance conferred by *acrAB* overexpression preceded high-level resistance mediated by target site mutation(s). Using AUC/MIC ~ 30, levofloxacin resistance emergence was delayed in Δ*acrAB* as compared to wild-type. In the absence of *acrAB*, *mdfA* and *norE* showed an increase in 2 out of 4 resistant isolates evaluated. Our data suggest that AcrAB efflux pump plays an essential role in facilitating the development of high-level resistance in Gram-negative bacteria. Our results also highlight that the importance of efflux pumps may have been under-appreciated till now.

Keywords: efflux pumps, AcrAB, levofloxacin, *Escherichia coli*

5.2 INTRODUCTION

Fluoroquinolones represent a widely prescribed class of antibacterial agents. They kill bacteria by targeting two essential DNA metabolic enzymes - DNA gyrase and topoisomerase IV, which are implicated in DNA supercoiling, untangling and unknotting during replication (Gellert et al., 1977; Zechiedrich et al., 1995; Khodursky et al., 1995; Deibler et al., 2001). Extensive use of the fluoroquinolones has contributed to a colossal increase in resistance (Lautenbach et al., 2004; Boyd et al., 2008) compromising the clinical utility of this class of drugs. Resistance to fluoroquinolones can be acquired by either chromosomally-mediated or plasmid-mediated mechanisms (Broskey et al., 2000; Cattoir et al., 2009). Chromosomally-mediated mechanisms are more commonly encountered in the clinical setting; bacteria may acquire mutation(s) at the target site where fluoroquinolones bind (*gyrA*/*gyrB* and *parC*/*parE*) or overexpression of genes encoding for efflux pumps. During the last decade, cases of plasmid-mediated quinolone resistance (plasmid encoding for genes such as *qnrA, aac*(*6′*)-*Ib*-cr, *qepA*) have also been reported (Martínez-Martínez et al., 1998; Jacoby et al., 2003; Morgan-Linnell et al., 2009).

Escherichia coli is the main aerobic commensal bacterial species in the human gastrointestinal tract. It is the leading cause of urinary tract infections (Kahlmeter, 2003) and intra-abdominal infections (Rossi et al., 2004). According to the European

Antimicrobial Resistance Surveillance, prevalence of fluoroquinolone resistance in *E. coli* has increased up to 50% in major parts of Europe (Kronvall, 2010).

In *E. coli,* it is known that at least three mutations in the 'quinolone resistance determining region' (QRDR) of the gyrase and topoisomerase IV genes are required for high-level fluoroquinolone resistance (Morgan-Linnell et al., 2007). It has also been reported that overexpression of the genes encoding for efflux pumps confer low-level resistance leading to only 2- to 8-fold increase in MIC values (Pumbwe et al., 2006; Chang et al., 2007; Kiser et al., 2010). A study by Morgan-Linnell et al. in clinical isolates of *E. coli* reported that 100% of the isolates had *gyrA* mutation(s), 85% had an additional *parC* mutation(s) and 33% had efflux pump (AcrA) overproduction (2009). Various other studies have similarly suggested the presence of both efflux pump overexpression and target site mutations in clinical isolates (Everett et al., 1996; Saito et al., 2006; Dunham et al., 2010). While efflux pumps do not appear to be as important as target site mutations in clinical isolates, various *in vitro* and *in vivo* studies using efflux pump mutant strains suggest that efflux pumps play a central role in the emergence of resistance (Lomovskaya et al., 1999; Kern et al., 2000; Jumbe et al., 2003). A study by Louie et al. suggested that efflux pumps might facilitate the acquisition of target site mutations in *Streptococcus pneumoniae* (Louie et al., 2007). The extent of contribution of efflux pumps and the temporal interplay between these two mechanisms in the development of high-level fluoroquinolone resistance is not well understood in Gram-

negative pathogens. In this study, we examined the temporal appearance as well as contribution of efflux pump (*acrAB*) expression and topoisomerase gene mutations to the emergence of levofloxacin resistance in wild-type *E. coli*.

5.3 MATERIALS AND METHODS

5.3.1 Antimicrobial agents

Levofloxacin hydrochloride was purchased from Waterstone Technologies (Carmel, IN). Moxifloxacin powder was a gift from Bayer Pharmaceuticals (West Haven, CT). A stock solution of the two drugs was prepared by dissolving the powder in sterile water and stored in aliquots at -70 °C. Prior to each investigation, an aliquot of the stock solution was thawed and diluted accordingly with cation-adjusted Mueller-Hinton broth (Ca-MHB) (BBL, Sparks, MD) or sterile water.

5.3.2 Microorganisms

Three strains of *E. coli* were studied. A standard wild-type *E. coli* - MG1655 and its two isogenic derivatives (*ΔacrAB* and *ΔacrR*) were investigated. In *ΔacrAB*, the major efflux pump mediating fluoroquinolone resistance in *E. coli* (i.e. *acrAB*) was deleted. In *ΔacrR,* the local repressor (*acrR*) was deleted causing stable overexpression of the *acrAB* efflux pump. The isogenic derivative strains were generous gifts from Dr. Lynn Zechiedrich,

Baylor College of Medicine, Houston, TX. Strain constructions and gene deletions have been described previously (Ma et al., 1996). These bacteria were stored at -70 °C in Protect storage vials (Key Scientific Products, Round Rock, TX). Fresh isolates were sub-cultured twice on 5% blood agar plates (Hardy Diagnostics, Santa Maria, CA) for 24 h at 35 °C prior to each experiment.

5.3.3 Susceptibility Studies

Minimum inhibitory concentrations (MICs) were determined in Ca-MHB by using a modified broth macrodilution method as described by the Clinical and Laboratory Standards Institute (CLSI, 2007). Serial two-fold dilutions of levofloxacin were prepared using Ca-MHB. Bacteria were grown in a sterile flask containing 10 ml Ca-MHB until visibly cloudy. The final concentration of bacteria in each broth macrodilution tube was diluted to approximately 5x 10^5 CFU/ml as measured by absorbance at 630 nm. MIC was read as the lowest concentration of the drug that resulted in no visible growth after 24 h of incubation at 35 °C. The experiments were repeated at least twice on separate days.

5.3.4 Mutational frequency

The mutation frequencies were determined by quantitatively plating overnight cultures of all three *E. coli* strains on the drug-free Mueller-Hinton agar II (MHA) (BBL, Sparks, MD) plates and MHA plates supplemented with levofloxacin at 4x their respective MIC.

After 72 h of incubation at 35 °C, the colonies were enumerated and the mutation frequencies were calculated by dividing the number of CFU/ml on the levofloxacin-supplemented agar plates by the number of CFU/ml on the drug-free agar plates.

5.3.5 Measurement of relative expression of *acrAB* efflux pump

To investigate the relative expression of efflux pump *acrAB*, a qRT-PCR method was used. We analyzed the relative expression of the *acrA* and *acrB* in three strains of *E. coli* - wild-type, Δ*acrAB* and Δ*acrR*. Total RNA was isolated using Qiagen RNeasy mini columns (Qiagen RNeasy kit, Valencia, CA). Reverse transcription to cDNA was performed using a GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA) with random hexamers. The DNA samples were analyzed in triplicate on an ABI PRISM 7000 (Applied Biosystems), using SYBR Green chemistry (Applied Biosystems, Foster City, CA). Relative quantification of the samples was performed with 16S *ribosomal RNA* (16S*rRNA*) as internal control and wild-type MG1655 was used as the reference strain. The changes in mRNA expression of *acrA* and *acrB* were quantified by the $\Delta\Delta C_t$ method, using a normalized expression analysis (Livak et al., 2001). No template controls were used as a negative control to detect any non-specific amplification. The sequences of the primers used are listed in Table C of the Appendix. In cases where the target was very abundant, the cDNA templates were diluted. It was recognized that SYBR Green lacks specificity and may bind to any double stranded DNA. To verify the product was pure, a dissociation curve was run with every analysis.

For selected isolates, relative expressions of *mdfA* and *norE* were performed by Michelle C. Swick from Baylor College of Medicine, Houston, TX.

5.3.6 Intracellular accumulation of fluoroquinolones

To demonstrate that change in the functional expression of *acrAB*, we analyzed the intracellular concentration of fluoroquinolones in all three strains of *E. coli*. The strains were incubated overnight under continuous shaking at 37 °C up to an A_{630} of approximately 0.85. Levofloxacin (10 μg/ml) was added and samples (20 ml) were taken at 5, 10 and 20 min. Samples were centrifuged (10,000x g) at 4 °C for 10 min. The pellets were washed twice with 50 mM sodium phosphate buffer (pH 7.0) and concentrated 20 times. Sonication was performed at 4 °C for 90 min followed by centrifugation (10,000x g) for 10 min at 4 °C. The supernatant collected was assayed by a validated LCMS/MS method as described below. In addition, intracellular accumulation of moxifloxacin was also measured at 20 min using identical conditions, to extend the generalizability of our results to other fluoroquinolones.

5.3.7 Hollow fiber infection model studies

This *in vitro* HFIM simulated human non-protein bound serum fluctuating drug concentration and allowed bacterial response to clinically relevant drug exposures to be examined. The schematics of the infection model have been described in detail

previously (Tam et al., 2005; Tam et al., 2007). The simulated half-life for levofloxacin was 5 to 7 h (Fish et al., 1997; Chien et al., 1997).

On the day of the experiment, a few colonies of bacteria were inoculated in Ca-MHB and incubated at 37 °C until reaching the log phase growth. The target inoculum was estimated based on absorbance at 630 nm and approximately 20 ml of bacteria (2x 10^5 CFU/ml) were introduced to the extracapillary space of the hollow fiber cartridge (FiberCell Systems, Inc., Frederick, MD). The experimental set up was maintained at 35 °C in a humidified incubator for the duration of the experiment. Levofloxacin was administered once daily to the HFIM (unless otherwise stated). To ascertain the pharmacokinetic profiles simulated in the infection models, serial samples (500 μl) were obtained in duplicate on different days from the circulating loop of the system. Levofloxacin concentration in these samples was assayed by a validated LCMS/MS method outlined below. A one compartment linear model was fit to the observed concentration-time profiles using the ADAPT II program (D' Argenio et al., 1997).

In addition, serial samples (500 μl) were obtained daily in duplicate (baseline, 4, 8, 24, 28, 48, 52 h and once daily thereafter; pre-dose when applicable) from the extracapillary space of hollow fiber cartridge to determine viable bacterial burden over time. To minimize the drug carryover effect, the samples were washed once with sterile saline. Before plating, bacterial samples were centrifuged for 15 min at 10,000x g and 4 °C.

Serial dilutions (10x) of samples were plated (50 µl) on drug-free MHA plates to quantify the total bacterial population. To quantify the resistant population, levofloxacin-supplemented agar plates were used. These agar plates were made with MHA containing levofloxacin at various multiples of MIC of the bacterial strain (e.g. 2x MIC, 4x MIC and 64x MIC) to detect isolates with different magnitude of reduced susceptibility (drug resistance). Since efflux pump overexpression confers a 2- to 6-fold increase in MIC, a 2x MIC plate was used to select isolates primarily with efflux pump overexpression. MHA plates supplemented with levofloxacin at 4x MIC and 64× MIC allowed us to more likely select isolates with single and double mutations in the DNA topoisomerases genes, respectively. Drug-free plates were incubated for 24 h and levofloxacin-supplemented MHA plates were incubated for up to 72 h (if required) at 35 °C before the colony-forming units were enumerated visually. The theoretical lower limit of detection was 400 CFU/ml.

5.3.7.1 Dose fractionation studies

Both AUC/MIC and C_{max}/MIC have been linked to the efficacy of fluoroquinolones (Wright et al., 2000). To ascertain the pharmacodynamic index most closely linked with resistance suppression for levofloxacin exposure, two sets of experiments using the same total dose (thus similar AUC) on different dosing schedules (thus different C_{max}) were utilized. For the first comparison, two HFIM systems were administered either once or

twice daily levofloxacin dosing resulting in different C_{max}/MIC (~ 0.042 μg/ml and 0.024 μg/ml), but over the period of 24 h had similar AUC/MIC (~ 13). For the second comparison, the same design was used. However, higher exposures of C_{max}/MIC (~ 0.144 μg/ml and 0.072 μg/ml) were investigated. These two systems had approximately similar AUC/MIC of 45.

5.3.7.2 Pharmacodynamic studies with wild-type *E. coli* and the mechanisms of resistance

To study the interplay of the two mechanisms of resistance in the wild-type strain, a once daily fixed dosing strategy was utilized initially. However, with this strategy we were unable to recover a high-level resistant bacterial sub-population (data not shown). The selective pressure exerted by the once daily dosing strategy was thought to be inadequate for the amplification of highly resistant isolates. Hence, in an attempt to select for high-level resistance, a sequentially escalating dose exposure was used. The dose exposure was increased every day, giving a higher selective pressure every 24 h to keep up with low to intermediate-level resistance developed over time.

Resistant isolates recovered from this experiment were re-tested for susceptibility to levofloxacin using an E-test (AB Biodisk, Piscataway, NJ). Sequencing and qRT-PCR analysis were performed (described in sections 5.3.9 and 5.3.5, respectively) to verify the

mechanism(s) of resistance. Since a transient increase in the expression of *acrAB* could be reversed when no selective antimicrobial pressure was present, these isolates were kept on levofloxacin-supplemented agar plates at the same concentration as they were initially plated, until the analyses were performed.

5.3.7.3 Pharmacodynamic studies comparing wild-type and Δ*acrAB* strains

To elucidate the impact of efflux pump deletion on resistance emergence, the wild-type and Δ*acrAB* strain were compared using HFIM. Since the preliminary susceptibility studies demonstrated that the Δ*acrAB* strain had a 4-fold reduction in MIC as compared to the wild-type strain, these comparisons were made in two ways. First, comparison was made using similar AUCs (~ 0.6 mg.h/L) for the two strains without adjusting for MIC difference. Second, comparison was made after adjusting for the MIC difference between the two strains, i.e. using similar AUC/MIC (~ 30). The resistant isolates recovered were evaluated for the mechanism(s) of resistance using sequencing and qRT-PCR analysis as described earlier.

5.3.8 LCMS/MS assay

An API 3200 Qtrap® triple quadrupole mass spectrometer (Applied Biosystems/MDS SCIEX, Foster City, CA) equipped with a TurboIonspray™ source was operated in positive ion mode to perform the analysis. The quantification was performed using

multiple reaction monitoring mode (MRM) with ion pair transitions to monitor levofloxacin and moxifloxacin (internal standard). The fragments for each compound detected were 362.0/318.0 (m/z) for levofloxacin and 402.0/384.0 (m/z) for moxifloxacin.

Ultra-performance liquid chromatography (UPLC) conditions for analysis were: Waters Acquity™ system and Acquity UPLC BEH C18 column (50x 2.1 mm I.D., 1.7 μm, waters, Milford, MA). A gradient elution was used with two mobile phases (mobile phase A - 0.1% (v/v) formic acid; mobile phase B – 100% acetonitrile). The flow rate used was 0.45 ml/min, column temperature was 45 °C and injection volume was 10 μl. To a 50 μl aliquot of sample, 10 μl of moxifloxacin (10 μg/ml) was added as internal standard, followed by 100 μl acetonitrile. The sample mixtures were vortexed for about 30 sec and precipitates were removed by centrifugation at 15,000x g for 15 min at room temperature. The supernatants were recovered and evaporated to dryness at 40 °C under air. The dry residue was reconstituted in 200 μl of 25% acetonitrile (v/v). A 10 μl aliquot of the resulting solution was injected onto the LC/MS/MS system for analysis. The assay was linear over the range of 0.008 μg/ml to 1.024 μg/ml ($r^2 \geq 0.996$). Samples expected to have higher concentration were diluted and samples expected to have lower concentrations were concentrated, before the assay. The intraday and interday coefficients of variation for the assay were 10% and 15%, respectively.

5.3.9 Gene amplification and sequencing

Isolates with reduced susceptibility were recovered from the levofloxacin-supplemented agar plates at various time points from the experiments conducted in the HFIM. To detect the mutations in the *gyrA* and *parC*, amplification and sequencing were performed.

QRDR of *gyrA* and *parC* were amplified by PCR. The Genbank accession number and sequences of primers designed to target the QRDR of these genes have been reported previously (Singh et al., 2009). Amplification was performed in a thermal cycler (Applied Biosystems, Foster City, CA) with an initial denaturing step of 94 °C for 10 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and final extension at 72 °C for 10 min. No template (negative) controls were included and the reactions were evaluated by electrophoresis in 2% agarose gel. The PCR products were sequenced by Lonestar laboratories, Houston, TX. Only QRDR of *gyrA* and *parC* were screened for mutation; these are the primary targets for fluoroquinolones in *E. coli*. Mutations in *gyrB* or *parE* do not consistently contribute to an increase in MIC and hence were not analyzed (Morgan-Linnell et al., 2009).

5.3.10 Statistical analysis

Relative expression of efflux pumps in resistant isolates recovered from HFIM and intracellular accumulation of isogenic derivatives (Δ*acrAB,* Δ*acrR*) were compared to the

wild-type strain (unless otherwise stated) using Student's *t*-test. *P*-values <0.05 were considered significant, unless otherwise stated.

5.4 RESULTS

5.4.1 Susceptibility studies

Levofloxacin MICs were 0.032 μg/ml for wild-type, 0.008 μg/ml for Δ*acrAB* and 0.064 μg/ml for Δ*acrR*. Disruption of *acrAB* has been shown to increase the susceptibility of various fluoroquinolones by 2- to 8-fold (Yang et al., 2003). Two-fold elevation in MIC in Δ*acrR* is consistent with the previous studies demonstrating low-level resistance with efflux pump overexpression (Pumbwe et al., 2006; Chang et al., 2007).

5.4.2 Mutational frequency

In all three *E. coli* strains investigated, mutational frequency was observed to be less than $1x\ 10^{-9.5}$. This supported that in the subsequent HFIM experiments, the bacterial burden studied (total population of $2x\ 10^{6}$ CFU) was homogenous and no pre-existing mutant was present.

5.4.3 Measurement of relative expression of *acrAB* efflux pump

The relative change in the expression of *acrA* and *acrB* in the three strains of *E. coli* are shown in Figure 5.1. The Δ*acrR* strain demonstrated a 2- to 3-fold increase in the expression of *acrAB* efflux pump relative to the wild-type strain.

5.4.4 Intracellular accumulation of fluoroquinolones

Intracellular fluoroquinolone concentration correlated with the relative expression of *acrAB* efflux pump. Intracellular accumulations of all three *E. coli* strains using levofloxacin and moxifloxacin at 20 min are shown in Figure 5.2. In comparison to wild-type, intracellular levofloxacin concentration was significantly higher in Δ*acrAB* ($P < 0.001$), which agreed well with the deletion of *acrAB*. Whereas in the Δ*acrR* deleted strain the intracellular concentration was lower ($P < 0.05$) as compared to wild-type, suggesting that overexpression of *acrAB* efflux pump in this strain could have caused the decrease in the drug concentration inside the bacterial cell (Figure 5.1). Both levofloxacin and moxifloxacin showed a similar pattern in the intracellular accumulation studies; however, the accumulation of moxifloxacin was almost one-third to that of levofloxacin (Figure 5.2). A bulky C-7 substitution in moxifloxacin has been suggested to reduce active efflux of the drug (Pestova et al., 2000). Our data suggests that while the bulky C-7 substitution makes the drug a poor substrate for efflux in Gram-positive bacteria, its effect in Gram-negative bacteria is less pronounced. In addition, Figure 5.3 shows the

intracellular accumulation at 4 time points over 20 minutes, to compare the extent of intracellular accumulation of levofloxacin in all three strains. The results suggested Δ*acrAB* had higher extent of accumulation than the wild-type and Δ*acrR* strains, at all the time points evaluated.

Figure 5.1: Relative expression of *acrAB* efflux pump in wild-type, Δ*acrAB* and Δ*acrR*. Data shown as mean ± standard deviation.

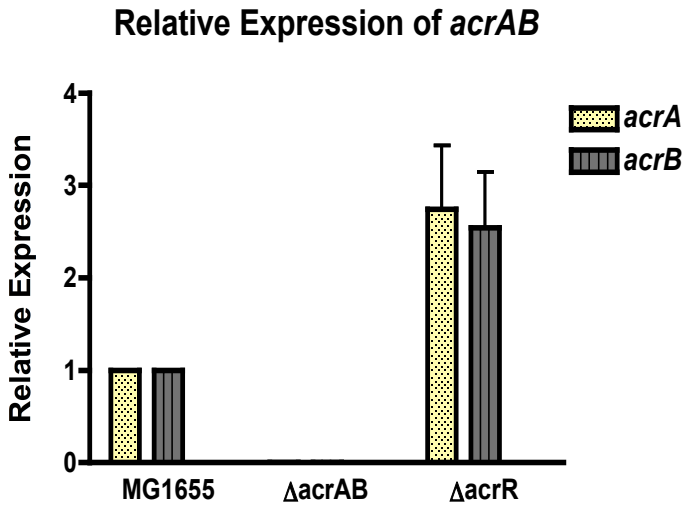


Figure 5.2: Intracellular accumulation of levofloxacin and moxifloxacin in wild-type, Δ*acrAB* and Δ*acrR* at 20 min. Data shown as mean ± standard deviation

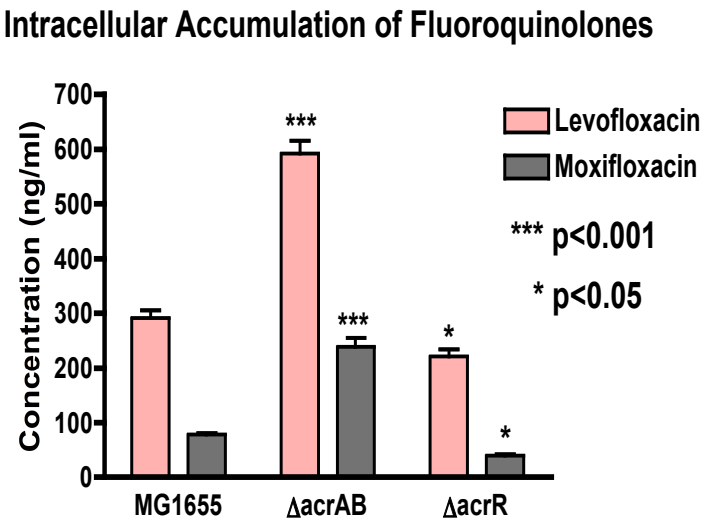
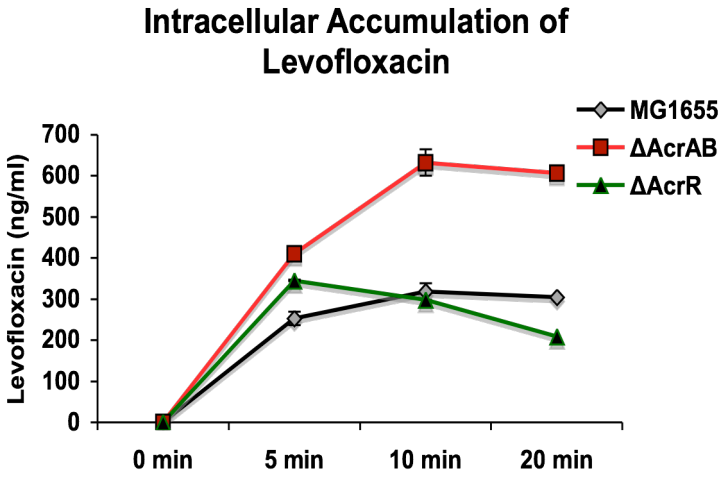


Figure 5.3: Intracellular accumulation of levofloxacin in wild-type, Δ*acrAB* and Δ*acrR* at 5, 10 and 20 min. Data shown as mean ± standard deviation.



5.4.5 Hollow fiber infection model studies

5.4.5.1 Pharmacokinetic studies

Satisfactory simulated levofloxacin exposures were achieved in all the HFIM conducted ($r^2 \geq 0.9$). Half-lives were within the target of 5 to 7 h (data not shown).

5.4.5.2 Dose fractionation studies

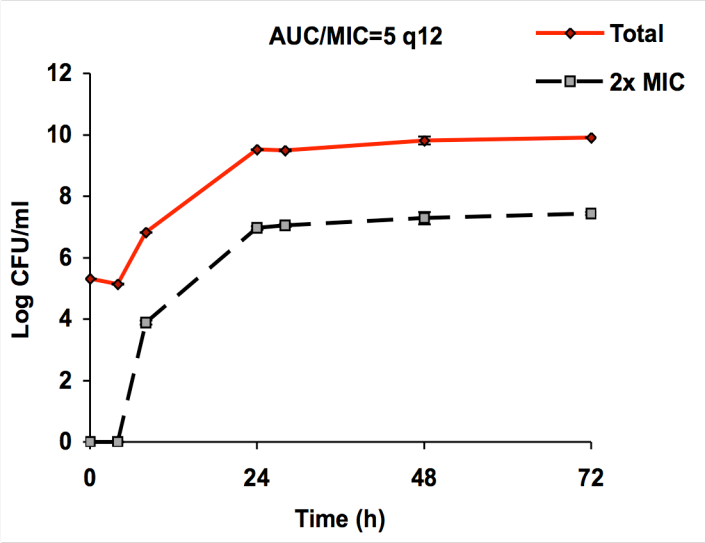
The results of dose fractionation studies to evaluate the PK/PD index of levofloxacin most closely linked to resistance suppression are shown in Figure 5.4. We expected that if C_{max}/MIC were the superior predictor, two different dosing schemes would have different C_{max} and hence should have different microbiological outcomes. However, if AUC/MIC was the better predictor, we should achieve the same microbiological outcome since similar AUC/MIC was targeted in the two dosing schemes. When an AUC/MIC ~ 13 was targeted, where once and twice daily dosing schemes were administered, the same microbiological outcome (regrowth) was observed, despite having different C_{max}/MIC (Figure 5.4 A and 5.4 B).

Similarly, using a higher exposure (AUC/MIC ~ 45), we observed the same microbiological outcome (suppression) in both systems (Figure 5.4 C and 5.4 D). Thus, our results suggested that AUC/MIC was the PK/PD index that was most closely linked

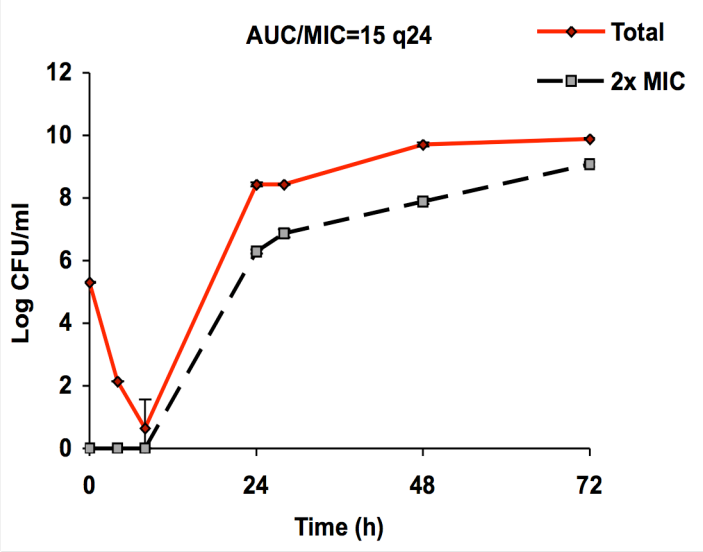
to resistance suppression for levofloxacin. Our results agree with previous reported results (Drusano et al., 2004) and thus once daily-dosing was used in the subsequent experiments.

Figure 5.4: Bacterial responses to levofloxacin in the HFIM using different C_{max}/MIC but similar daily AUC/MIC. Regrowth was observed in both A and B and suppression was observed in C and D. Data shown as mean ± standard deviation.

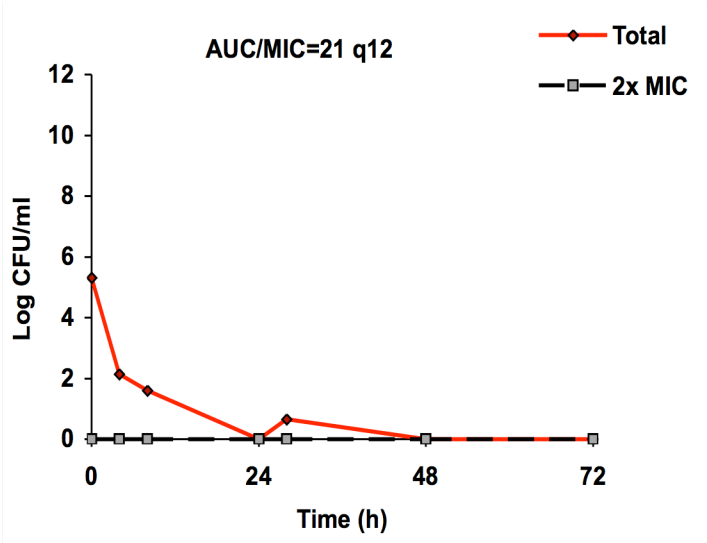
A



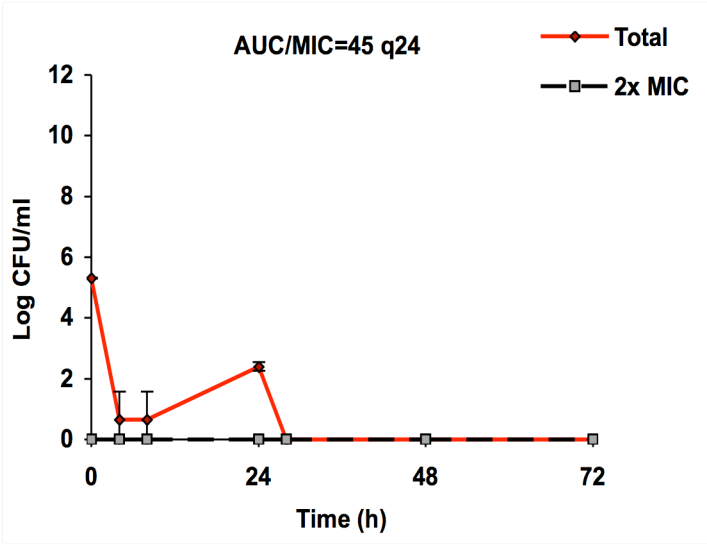
B



C


AUC/MIC=21 q12
Total
2x MIC
Log CFU/ml
12
10
8
6
4
2
0
0
24
48
72
Time (h)

D


AUC/MIC=45 q24
Total
2x MIC
Log CFU/ml
12
10
8
6
4
2
0
0
24
48
72
Time (h)

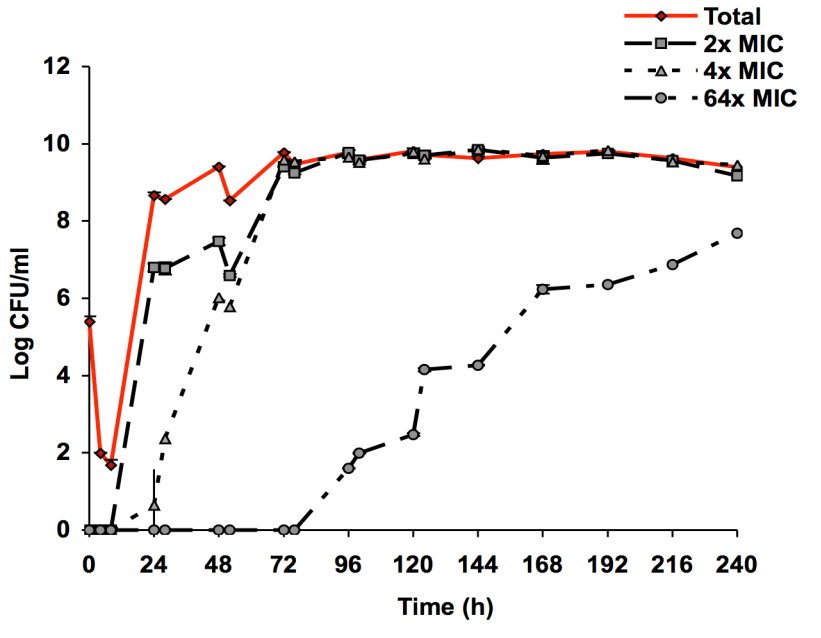
5.4.5.3 Pharmacodynamic studies with wild-type *E. coli* and the mechanisms of resistance

Bacterial response to sequentially escalating levofloxacin exposures is shown in Figure 5.5. The AUC/MIC achieved in this experiment is shown below in Figure 5.5 and ranged approximately from 7 to 3200. As expected, regrowth was observed and the higher selective pressures exerted by the sequentially escalating dosing strategy led to an increase in the resistant population (recovered at 64x MIC levofloxacin-supplemented agar plates).

To study the temporal interplay of mechanisms of resistance, isolates were recovered from levofloxacin-supplemented agar plates at three different time points - early (24 h), intermediate (120 h) and late (240 h). These recovered isolates were re-tested for levofloxacin susceptibility. Results of these susceptibility studies and sequencing analysis are shown in Table 5.1. The qRT-PCR analysis for *acrAB* efflux pump in resistant isolates revealed at least three distinct profiles (Figure 5.6). The resistant isolates recovered early (24 h) had primarily 2-6x MIC elevation and no point mutation; these early resistant isolates had a 2- to 8-fold increase in *acrAB* expression. High-level (≥ 64x MIC) resistant isolates recovered from an intermediate time point (120 h) were found to have target site mutation at *gyrA* (S83L); the relative expressions of *acrA* and *acrB* were still high (2- to 8-fold). The isolates recovered at a later time point (240 h) had ≥ 100-fold increase in MIC with point mutation(s) (*gyrA* S83L +/- *parC* E84K) in QRDR.

Regression of *acrAB* overexpression was observed when these isolates were compared to isolates recovered at 120 h ($P < 0.05$), suggesting that the *acrAB* efflux pump was transiently overexpressed in these isolates. These results suggested efflux pump overexpression played an essential role in early stages of resistance development. Relative expressions of *mdfA* and *norE* efflux pumps were also analyzed in the resistant isolates recovered (data not shown). An increasing trend was observed in the relative expression of these efflux pumps over time, however this increase was not statistically significant.

Figure 5.5: Bacterial response to sequentially escalating levofloxacin exposures in HFIM for wild-type *E. coli*. Data shown as mean ± standard deviation

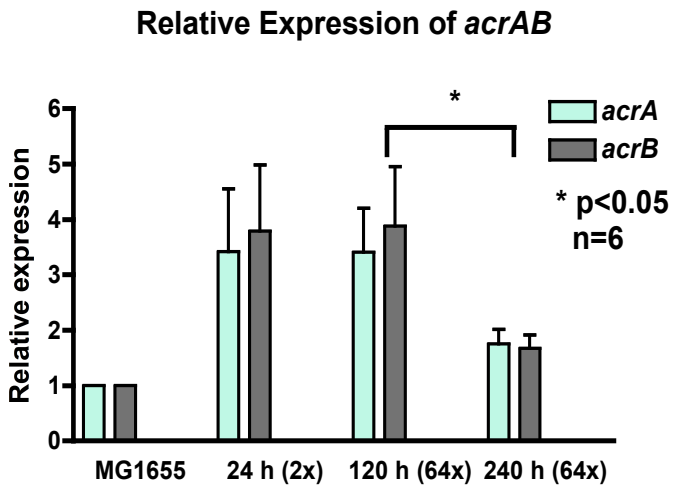


Time (h)	0-24	24-48	48-72	72-96	96-120	120-144	144-168	168-192	192-216	216-240
AUC/MIC	7	17	57	146	340	577	577	577	1692	3172

Table 5.1: Levofloxacin MIC fold elevation and mutations present in *gyrA* and *parC* in resistant isolates recovered from HFIM studies with wild-type *E. coli*. (ND = not detected)

Isolates	MIC fold elevation	*gyrA* mutation	*parC* mutation
24 h_2x_a	4	ND	ND
24 h_2x_b	4	ND	ND
24 h_2x_c	4	ND	ND
24 h_2x_d	6	ND	ND
24 h_2x_e	3	ND	ND
24 h_2x_f	6	ND	ND
120 h_64x_a	188	S83L	ND
120 h_64x_b	188	S83L	ND
120 h_64x_c	125	S83L	ND
120 h_64x_d	125	S83L	ND
120 h_64x_e	47	S83L	ND
120 h_64x_f	63	S83L	ND
240 h_64x_a	250	S83L	ND
240 h_64x_b	188	S83L	E84K
240 h_64x_c	125	S83L	ND
240 h_64x_d	188	S83L	ND
240 h_64x_e	188	S83L	ND
240 h_64x_f	188	S83L	ND

Figure 5.6: Relative expression of *acrAB* in resistant isolates recovered from HFIM studies in wild-type *E. coli* at 3 time points – 24 h, 120 h and 240 h. Data shown as mean ± standard deviation.



5.4.5.4 Pharmacodynamic studies comparing wild-type and Δ*acrAB* strains

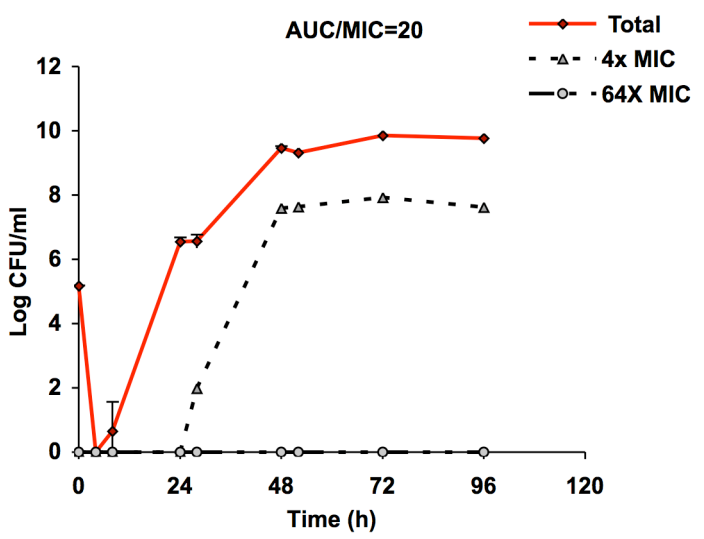
To elucidate the impact of efflux pump on resistance emergence, the wild-type and Δ*acrAB* strains were compared in the HFIM. These strains were compared in two ways, first using similar AUCs and later using similar AUC/MIC. As shown in Figure 5.7, similar levofloxacin AUCs (AUC ~ 0.6 mg.h/L for wild-type), without adjusting for MIC decrease in Δ*acrAB*, resulted in suppression of resistance in Δ*acrAB* (AUC/MIC = 72), and regrowth in wild-type (AUC/MIC = 20). This suggested that if dose equivalent to levofloxacin exposure of AUC/MIC = 20 were to be co-administered with agents inhibiting AcrAB efflux pump, suppression of resistance could be achieved instead of regrowth with levofloxacin alone. Subsequently, comparison was done after adjusting for the MIC difference between the two strains. An exposure-related relationship was observed in both strains, with respect to resistance emergence. However, when an AUC/MIC ~ 30 was investigated in both strains, we found a 72 h delay in emergence of resistance in Δ*acrAB* as compared to the wild-type (Figure 5.8).

Resistant isolates recovered at 120 h from levofloxacin-supplemented agar plates in the Δ*acrAB* experiment (AUC/MIC = 30) were also analyzed for the mechanisms of resistance. All the isolates analyzed had 8- to 16-fold MIC elevation as shown in Table 5.2. Our results revealed a mixed population with *gyrA* (S83L) mutation in 2 out of 4 isolates. Since the *acrAB* efflux pump was deleted in these isolates, we investigated the

relative expression of two other efflux pumps (*mdfA* and *norE*) implicated in mediating fluoroquinolone resistance in *E. coli*. Four isolates analyzed were grouped into two - with *gyrA* mutation or without *gyrA* mutation for analysis. Relative expression of the *mdfA* and *norE* efflux pumps in these two groups of resistant isolates is shown in Figure 5.9. The isolates with no mutation in *gyrA* or *parC* had significant increase in the expression of *norE* as compared to wild-type *E. coli*. An increase in *mdfA* efflux pumps was also observed, however it was not significant.

Figure 5.7: Bacterial responses to similar levofloxacin AUC exposures (AUC ~ 0.6 mg.h/L) in HFIM studies for *E. coli* strain - wild-type (A) and Δ*acrAB* mutant (B). Data shown as mean ± standard deviation.

A



B

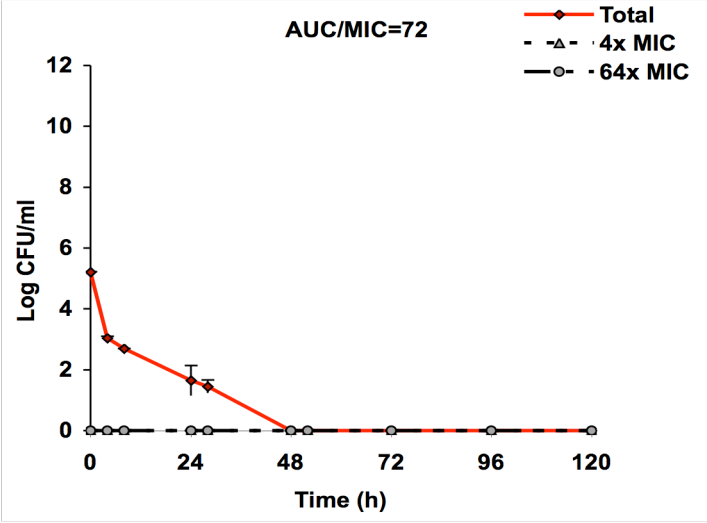
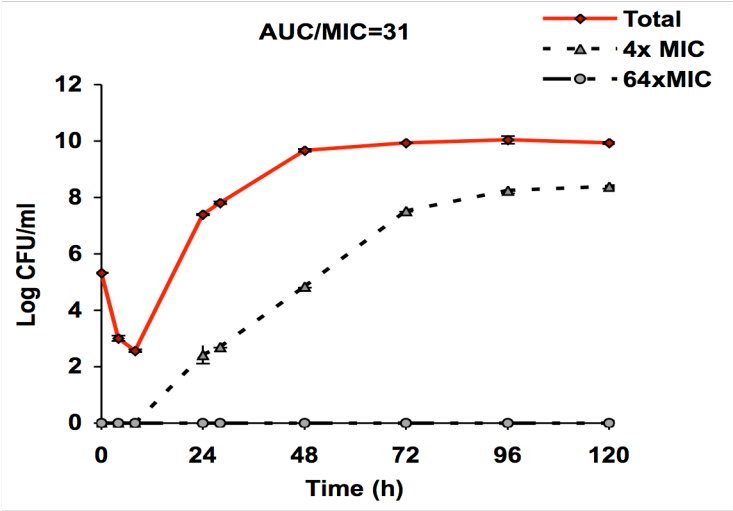


Figure 5.8: Bacterial responses to almost identical levofloxacin exposures (AUC/MIC ~ 30) in HFIM studies for *E. coli* strains - wild-type (A) and Δ*acrAB* mutant (B). Data shown as mean ± standard deviation.

A



B

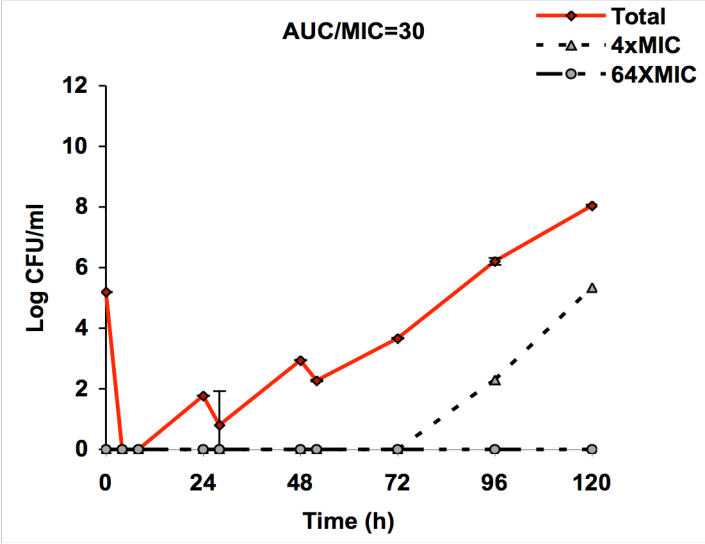
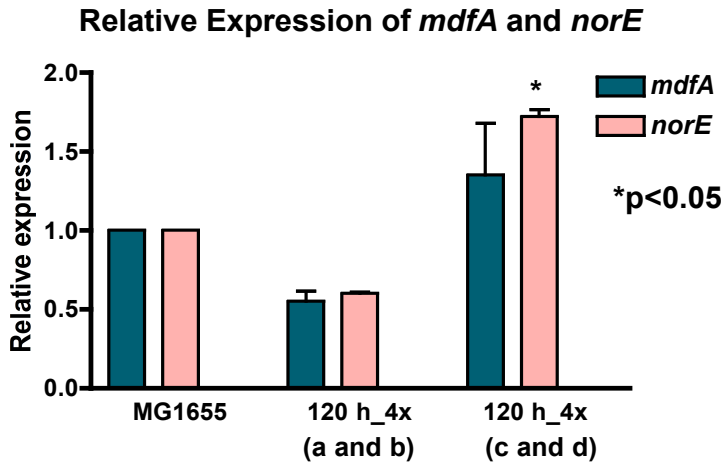


Table 5.2: Levofloxacin MIC fold elevation and mutations in *gyrA* and/or *parC* in resistant isolates recovered from HFIM studies with Δ*acrAB*. (ND = not detected)

Isolates	MIC fold elevation	*gyrA* mutation	*parC* mutation
120 h_4x_a	16	S83L	ND
120 h_4x_b	16	S83L	ND
120 h_4x_c	8	ND	ND
120 h_4x_d	16	ND	ND

Figure 5.9: Relative expression of *mdfA* and *norE* efflux pumps in resistant isolates derived from HFIM experiment with Δ*acrAB* (by Michelle C. Swick from Baylor College of Medicine, Houston, TX.). Resistant isolates were grouped in two- with mutation (4x_a and 4x_b) and without mutations (4x_c and 4x_d) for analysis. Data shown as mean ± standard deviation.



5.5 DISCUSSION

Several surveillance studies have showed a temporal rise in fluoroquinolone resistance in recent years (Cattaneo et al., 2008; Hawkey et al., 2009). Resistance has increased up to 50% in some part of the world leading to a conundrum with the usage of this class of drug (Kronvall, 2010). Overall, increasing resistance against fluoroquinolones has jeopardized their clinical utility.

A study done by Jumbe et al. showed efflux pumps played a central role in fluoroquinolone resistance emergence in *S. pneumoniae* (2003). When efflux pump overexpressed strain was inoculated in a nonneutropenic murine thigh infection model, a dramatic increase in mutants resistant to levofloxacin was observed ($<1/10^{8.5}$ to approximately $1/10^{4.5}$), as compared to wild-type *S. pneumoniae*. These findings led the authors to conclude that efflux pump overexpression increases the likelihood of target site point mutations. Further, using an *in vitro* HFIM, Louie et al. compared the effect of efflux pump inhibitor (reserpine) on resistance emergence in a wild-type *S. pneumoniae* and its efflux pump overexpressed isogenic strain (2007). In their study, levofloxacin (500 mg, QD) lead to resistance emergence in both strains. However, when levofloxacin was combined with reserpine, resistance suppression was achieved in both the wild-type and the efflux pump overexpressed strain.

Taken together, the work by Jumbe et al. and Louie et al. demonstrated the interplay and facilitation between the two mechanisms of fluoroquinolone resistance. However, both studies mentioned above were carried in a Gram-positive pathogen - *S. pneumoniae*. While efflux pumps mediate low-level resistance in both Gram-positive and Gram-negative bacteria, their effect is more robust in the latter case due to two reasons. First, the presence of outer membrane in Gram-negative bacteria, significantly slows down the entry of both lipophilic and hydrophilic drugs. Second, the presence of tripartite efflux pumps (such as AcrAB-TolC, MexAB-OprM) directly extrudes the substrates into the external medium leading to decreased amounts of drugs not only in the cytoplasmic but also in the periplasmic space (Lomovskaya et al., 2001). This is also consistent with various reports stating that a higher AUC/MIC ratio is required for suppressing fluoroquinolone resistance in Gram-negative bacteria when compared to Gram-positive pathogens (Craig, 2001; Jacobs, 2001; Levison et al., 2009).

Our study provides insights into the temporal interplay of the two main mechanisms of fluoroquinolone resistance in a clinically important Gram-negative pathogen - *E. coli.* In this study, we analyzed resistant isolates at various time points to investigate the temporal pattern of appearance of efflux pump overexpression and target site mutations. Our results suggest that in wild-type *E. coli,* early low-level levofloxacin resistance conferred by *acrAB* overexpression precedes high-level resistance mediated by target site

mutation(s). Several studies in fluoroquinolone-resistant clinical isolates of *E. coli* have suggested overproduction of efflux pumps (Chang et al., 2007; Morgan-Linnell et al., 2009). According to these studies, efflux pumps contribute little (~30% - 50%), compared to mutations in DNA topoisomerases. However, these studies usually do not describe at what time during the course of infection these isolates were collected. Our results suggest that time is a very crucial factor in resistance mediated by efflux pumps. The prominent increase in *acrAB* expression at early time point and then regression at later time point suggests, if clinical isolates are collected at later time point they may actually fail to capture this transient but important overexpression of efflux pumps. It might be possible that the remaining clinical isolates had an overexpression of efflux pump but later regressed back to normal constitute levels. These results highlight that the importance of the efflux pump may have been under-appreciated. Our results provide a framework for the interplay of the two mechanisms of resistance and the quintessential role mediated by these efflux pumps in this interplay.

This time delay in resistance emergence observed in Δ*acrAB* could be utilized clinically by using a 'hit hard and early' dosing strategy to suppress resistance, when levofloxacin is co-administered with an efflux pump inhibitor. In this strategy, a higher dose exposure is given early on during infection to suppress resistance emergence. In contrast to the complete suppression obtained by Louie et al. against *S. pneumoniae,* using reserpine, our results displayed only a 3-day delay in resistance emergence with the efflux pump deleted

strain. These results are not surprising considering that the efflux pumps in Gram-negative bacteria are more diverse and difficult to overcome. Another reason could be that reserpine is a non-specific efflux pump inhibitor whereas in our study we deleted only one efflux pump. As the molecular studies in resistant isolates recovered from Δ*acrAB* experiments suggest, other efflux pumps (*mdfA* and *norE*) may compensate for the lack of *acrAB,* eventually leading to target site mutation(s).

As mentioned above, the major limitation of this study was the deletion of only one efflux pump. A study by Yang et al. compared the MIC change between *E. coli* mutants with deletion of *acrAB* alone and deletion of all three efflux pumps mediating fluoroquinolone resistance and found no difference (Yang et al., 2003). Considering these results and that AcrAB is the major efflux pump implicated in fluoroquinolone resistance in *E. coli*, deletion of genes encoding for this efflux pump seemed to be the logical first step. However, as suggested by our results, other efflux pumps might compensate. A possible future strategy could be inhibition or deletion of all three efflux pumps implicated in the efflux of fluoroquinolone. It should also be noted that only the *gyrA* and *parC* were sequenced and the possibility of mutations in *gyrB* and *parE* cannot be ruled out. Another limitation was the lack of an immune component in this *in vitro* infection system.

Various studies have emphasized the role of efflux pumps in resistance development in

different pathogens. Apart from the AcrAB-TolC efflux pump in *E. coli,* multidrug resistance has been attributed to the MexAB-OprM efflux pump in *Pseudomonas aeuroginosa* and AdeABC in *Acinetobacter baumanii* (Hocquet et al., 2003; Ruzin et al., 2007). The implication of efflux pumps as a major causative factor in antibiotic resistance to virtually all classes of antibiotics and its association with multidrug resistant phenotypes makes them an important target. A strategy to overcome the potent effect of these multidrug resistant efflux pumps seems promising for fighting resistance development. Our results highlight the importance of *acrAB* in the development of fluoroquinolone-resistance, and suggest that inhibiting efflux pump could be a robust strategy for combating this worldwide problem of antibiotic resistance. These results also suggest that while efflux pump inhibition is an attractive target, it is also incredibly challenging owing to the variability in different pathogens and the inherent compensation within the pathogen. Clinical relevance of our findings warrants further *in vivo* investigations.

CHAPTER 6

Conclusions

"There is probably no chemotherapeutic drug to which in suitable circumstances the bacteria cannot react by in some way acquiring 'fastness' [resistance]."

- Alexander Fleming, 1946

How bacteria survive an antimicrobial pressure presents a very intriguing question in science. Our results have added to the existing understanding of the process of antibiotic resistance development in bacteria. In all of the experiments conducted using the hollow fiber infection model, whenever regrowth was observed in the wild-type *E. coli*, the resistant isolates recovered at an early time point had *acrAB* overexpression and no point mutation. This increase in *acrAB* expression was irrespective of once daily or sequentially escalating dosing strategy; highlighting the importance of efflux pumps in the development of antibacterial resistance. As the process of resistance development progressed, it became more complicated. At the intermediate time point evaluated (120 h), over-production of efflux pump (or overexpression of the genes encoding the AcrAB efflux pump) worked in concert with single point mutation in *gyrA* to cause high-level

resistance. This acquisition of high-level resistance by only one point mutation in *gyrA* was surprising, considering earlier studies in clinical isolates have suggested multiple mutations in DNA topoisomerases of high-level resistant isolates (Everett et al., 1996; Morgan-Linnell et al., 2009). One possible explanation for this discrepancy could be the presence of other mutations within the target genes but outside of *gyrA* and *parC;* such as *gyrB* or *parE*. However, since the above-mentioned studies also suggested that *gyrB* and *parE* mutations do not consistently contribute to an increase in MIC; this explanation seems inadequate (Everett et al., 1996; Morgan-Linnell et al., 2009).

Another explanation could be mutations in non-target genes, possibly in genes regulating efflux pump expression. This explanation is supported by various studies (Kern et al., 2000; Jellen-Ritter et al., 2001). In their study, Kern et al. examined *E. coli* mutants selected stepwise from agar plates supplemented with ofloxacin at various multiples of MIC (2000). They reported that the first-step mutants invariably had a single mutation in *gyrA*. The second-step mutants had non-target gene mutations including but not limited to mutations in *mar* (multiple antibiotic resistance) locus. Extending this work, Jellen-Ritter et al. demonstrated that mutations in *acrR* could be one of the possible mechanisms in the isolates where *mar* mutations were not present (2001). In our study, we did not sequence the *acrR* or the *mar* locus and hence this possibility cannot be ruled out. However, the regression in *acrAB* expression in resistant isolates recovered at 240 h suggests that mutation(s) in these genes was not the only possibility. It could be possible that in

isolates with high-level resistance to fluoroquinolones, the bacterial population is divided into sub-populations with different levels of expression of efflux pumps. Some isolates might acquire mutations in the genes regulating these efflux pumps and would have stable overexpression of efflux pump(s). Other isolates might overproduce efflux pump(s) transiently, returning back to their normal constitutive level after some time. These transiently over-produced efflux pumps might take turns within themselves, so when the expression of one efflux pump returns towards baseline level, other efflux pump(s) might take over. In our study, we did see an increase in the expression of *mdfA* and *norE* when *acrAB* regressed, however this increase was not significant. Among the isolates we analyzed, we did not see any particular pattern on an individual isolate level, i.e., when all three efflux pumps were analyzed in each isolate, a decrease in *acrAB* was not necessarily associated with an increase in *mdfA* or *norE*. This was even more intriguing, suggesting the possibility that the expression of efflux pumps might be coordinated on a sub-population or group level. It could be possible that when one group has regression in the *acrAB* efflux pump, another group might overexpress *mdfA* or *norE*. Although, on an overall population level, this process may or may not be reflected in terms of an increase in MIC, it would provide an entire population a strategy to survive and would help in an efficient use of energy that is required for the efflux of these antimicrobial agents.

Similar temporal studies have also been conducted for the SOS response in *E. coli* and have revealed an intriguing dynamic property associated with this phenomenon (Friedman et al., 2005). This study was done using fluorescent reporter genes inserted on plasmids into individual *E. coli* cells to measure the promoter activities of several genes involved in the SOS response system. Under UV irradiation, the promoter activities of both *recA* and *lexA* increased after a short delay, and reached peak values after approximately 30 min. If the irradiation was sufficiently strong, a second peak appeared after 60 - 80 min and a third peak after 90 - 130 min. These results suggested that stress response systems such as SOS might operate in a wave-like pattern at predefined time intervals. This study also suggested that the frequency of this stress response is dependent on the intensity of the stimulus and a second or third peak only occurred when the stimulus was strong enough. Previous work by Tam et al. showed that the relationship between quinolone exposures and resistance amplification is characterized by an inverted U phenomenon (2007). In this study the authors enumerated the resistant population obtained from exposing *Klebsiella pneumoniae* to different escalating AUC/MIC exposures. They demonstrated that the log CFU/ml of resistant isolates increased as the AUC/MIC exposure increased but eventually led to a complete suppression at higher AUC/MIC. The expression of efflux pumps might work in a similar manner.

Our *in vitro* HFIM results with the Δ*acrAB* strain added another level of complexity. Using similar AUC/MIC exposure in Δ*acrAB* and wild-type strain, there was a delay in

the emergence of resistance in Δ*acrAB* as compared to the wild-type strain. The resistant isolates recovered had an 8- to 16-fold increase in MIC values. The increase in *norE* expression was significant in two isolates when compared to the wild-type strain. This suggested that efflux pumps like *mdfA* and *norE*, which did not play a significant role when *acrAB* was present, might become significant when *acrAB* is absent. These findings are similar to that obtained by Lomovskaya et al., where they studied isogenic strains of *P. aeruginosa* lacking individual efflux pumps (MexAB-OprM, MexCD-OprJ, MexEF-OprN) and strains with various combination of mutations in efflux pump and target site for fluoroquinolones (1999). A stepwise selection of mutants on levofloxacin-supplemented plates revealed that lack of one efflux pump was compensated by over-production of other efflux pump(s). The authors did not obtain isolates with target site mutation when triple knockout strain of efflux pumps was evaluated. They also demonstrated that deletion of *oprM* led to 8- to 16-fold reduction in the MIC of strains with either one (*gyrA*) or multiple mutations (two *gyrA* and one *parC*) in the target sites. These results suggested that efflux pumps might contribute to antibiotic resistance even in the presence of target site mutation(s).

This diversity and extensive "teamwork" exhibited by efflux pumps has been a roadblock in the development of efflux pump inhibitors. In a given bacterial species, usually there is a combination of multiple broad substrate efflux pumps and some substrate-specific efflux pumps. An attempt to develop a highly specific efflux pump inhibitor of MexAB-

OprM failed due to acquisition of a *mexB* mutation (gene encoding for MexB component of this efflux pump), leading to resistance to these agents (Lomovskaya et al., 2006). Another efflux pump inhibitor with broad substrate specificity was abandoned due to issues associated with pharmacokinetics and toxicity of the agent (Watkins et al., 2003). These examples indicate the daunting challenge presented in trying to develop efflux pumps as targets. It is expected that successful efflux pump inhibitors might potentiate the activity of various clinically available antibiotics and minimize the development of resistance to the co-administered agents (Ng et al., 1994; Westbrock-Wadman et al., 1999; Vogne et al., 2004).

The *in vitro* model utilized in our studies could not mimic *in vivo* conditions such as the pathology of infection, host-defense mechanisms, virulence and metabolic behavior of the pathogen. Thus, the clinical relevance of these findings warrants further *in vivo* investigation. Some previous studies have suggested beneficial effects of inhibiting/deleting efflux pumps *in vivo*. Using an *acrAB* deleted strain of *Klebsiella pneumoniae*, Padilla et al. reported a decrease in the capacity of this strain to cause pneumonia, indicating decrease in virulence (2010). They also suggested that AcrAB efflux pump mediated resistance against antimicrobial peptides present in the lungs (e.g. human neutrophil defensin-1 and human β defensin-1). These antimicrobial peptides are considered the first barrier presented by the innate immune system against infections. They concluded that in addition to imparting a multidrug resistance phenotype, the

AcrAB efflux pump might represent a novel virulence factor required to resist innate immune defense mechanisms of the lungs, thus facilitating pathogenesis. Studies have also suggested a role of these efflux pumps in transporting virulence determinants such as adhesions, toxins and other proteins essential for colonization and invasion of the host cells (Bina et al., 2001; Piddock, 2006a). Efflux pumps have also been implicated in bacterial fitness and pathogenicity. Two studies have suggested that the over-production of the MtrC-MtrD-MtrE efflux pump in *Neisseria gonorrhoeae*, by mutation in the repressor (*mtrR*), was associated with increased bacterial fitness (Warner et al., 2008; Warner et al., 2007). Another study by Webber et al. suggested that deletion of each component of AcrAB-TolC decreased the expression of numerous genes encoding for proteins involved in pathogenicity (2009). TolC and its homologues have been reported to play an important role in bacterial survival, and possibly confer virulence during infection of the host (Andersen et al., 2000). In a study evaluating the expression of efflux pumps in fluoroquinolone-susceptible and -resistant clinical isolates of *E. coli,* Swick et al. reported the order of abundance of efflux pump transcripts in all fluoroquinolone-susceptible isolates as *tolC* > *acrA/B* > *mdfA/norE* (2010). This abundance of *tolC* might be an indicative of functions beyond efflux of antibiotics. These studies suggest that efflux pumps may contribute to more than multidrug resistance and highlight the importance of efflux pumps.

Our findings have also pointed to a major concern in clinical settings. Usually in the case of infections, it takes 24 to 48 h for the clinical microbiological laboratory to confirm the microorganism and empiric treatment is given in the meantime. Results from our study suggest that the initial 24 h are crucial in antimicrobial resistance emergence. During this early period, there is only a 2- to 6-fold increase in MIC and hence resistance emergence can be overcome easily as compared to a later time when a 100- to 300-fold increase in MIC occurs and the antimicrobial agent becomes ineffective. Thus, with appropriate treatment at very early stages of infection, it might be more likely to restrict resistance emergence.

The extent of impact of efflux pumps on resistance emergence can be further evaluated using various approaches. The first approach would be to evaluate the effect of a triple efflux pump mutant (Δ*acrAB*Δ*mdfA*Δ*norE*) on resistance emergence, using an *in vitro* HFIM. This would give an idea about the resistance development process in the absence of all three efflux pumps mediating fluoroquinolone resistance. It would be interesting to know if some other compensatory mechanisms take place in the event of deletion of all three efflux pumps. Perhaps, it would be more fascinating to look into the molecular changes in the resistant isolates obtained in these studies, by sequencing their entire genome. Sequence comparison of early time point (24 h) and late time point (240 h) resistant isolates from wild-type strain could reveal gene mutations involved in the resistance development process. DNA microarray experiments can also be done to

investigate the difference in the expression of various genes in resistant isolates with transient versus stable overexpression of the efflux pumps. This might suggest the molecular mechanisms involved in the transient overexpression of the efflux pumps. Further, comparing these wild-type resistant isolates to the resistant isolates (if any) recovered from experiments performed on the efflux pump deleted strains ((Δ*acrAB*Δ*mdfA*Δ*norE* and/or Δ*acrAB*), would suggest about the probable compensatory mechanisms operating in the event of efflux pump deletions. A common concern in these knockout strains is the change in the pleiotropy of the microorganism that comes with the deletion of the genes. Efflux pump inhibitors can also be used for the evaluation of the effect of efflux pumps on resistance emergence, however there are two major concerns with such inhibitors. First, some of these inhibitors have been found to be substrates of efflux pumps themselves, thus questioning their credibility (Lomovskaya et al., 2006). Second, the concentrations required for efflux pump inhibitory activity are often associated with toxicity and hence, are not clinically relevant (Schmitz et al., 1998).

Further, *in vivo* experiments should also be performed using the wild-type and the efflux pump knockout strains to evaluate the impact of efflux pumps on resistance emergence. This will suggest about the effect of deletion of efflux pumps on dose exposure as well as on virulence, immune components and fitness of the bacteria. As stated earlier, it has been suggested that TolC – the outer membrane channel protein, is also implicated in pathogenesis and virulence, and it would be interesting to study the impact of *tolC*

deletion in animal models of infection. In clinical settings, mixed infections could be present. While, only one Gram-negative bacterial species has been evaluated in our study, it is imperative to investigate other bacterial species to increase the clinical relevance of our results. A potent efflux pump inhibitor can be utilized to study the effect of efflux pumps in multiple bacterial species. Thus, a clinically successful efflux pump inhibitor should not only be effective against multiple efflux pumps in one bacterial species but also against efflux pumps in more than one bacterial species.

Our study using *in vitro* HFIM represented only the direct relationship between the drug exposure and the microorganism. In humans, the antibiotic may not always reach the site of infection in the dose exposure desired. Antibiotic tissue penetration depends on properties of the antibiotic (e.g. lipid solubility, molecular size) and tissue (e.g. adequacy of blood supply, presence of inflammation). In acute infections, antibiotic tissue penetration is not always problematic due to increased microvascular permeability from the local release of inflammatory mediators. In contrast, chronic infections (such as chronic osteomyelitis, chronic prostatitis) often rely on only the chemical properties of the drug for tissue penetration. Antibiotic cannot be expected to eradicate organisms from areas that are difficult to penetrate or when the pathogen is preferentially intracellular or have impaired blood supply (such as abscesses). This inadequate penetration of antibiotic at the infection site can lead to a lower drug exposure than actually desired and eventually resistance emergence. Other factors that complicate the clinical scenario are –

the presence of plasmids and the presence of bacterial population with different susceptibilities. The presence of plasmids leads to horizontal transfer of genes encoding for resistance and hence the bacteria become insensitive to the drug. Sometimes a mixed population of susceptible and resistant bacteria may exist because of prior inadequate drug exposure or a higher inoculum of bacteria. Since this mixed population will have different MICs, different AUC/MIC will be achieved against both the populations. In this case, it may be possible that the susceptible bacteria get killed and the resistant one survive, leading to resistance emergence over time. The emergence of antibiotic resistance is also affected by the presence or absence of a functional immune response in the human being. In immunocompetent individuals, a lower drug exposure is required since host defense system aids in clearing tissues of the infecting microorganism. In cases where the host defense system is inadequate (e.g. agranulocytosis) or the host defense system is impaired locally at the site of infection (e.g. cardiac vegetation in left-sided endocarditis, cerebrospinal fluid in meningitis) the pathogen resumes growth, as the drug concentration decreases, and the infection relapses. Bacterial infection in these circumstances will require higher concentration of the antimicrobial agent. The presence of a foreign body may also adversely influence the effectiveness of an antimicrobial agent. The foreign body acts as a nidus on which microorganisms may grow as a biofilm. A biofilm is a community of microorganisms embedded in a matrix secreted by the microorganisms, which helps them attach to other bacteria, host cells, or foreign objects

and which shields them against host defense system and penetration by many antimicrobial drugs (Stewart et al., 2001).

In summary, resistance emergence presents a debilitating challenge in the management of infectious diseases. Data from this dissertation add to our understanding of resistance development and the importance of dose exposure in resistance suppression. Temporal interplay between the two main mechanisms of fluoroquinolone resistance suggests resistance development in bacteria operates in a highly coordinated manner and its regulation is multi-factorial. Our findings suggest that the inhibition of efflux pumps could be a potential strategy to thwart the problem of antibiotic resistance. Considering the genetic plasticity in the bacteria, these findings just seem to be the tip of the iceberg and much needs to be unraveled in the future.

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APPENDIX

Table A: Forward and reverse primers for *gyrA* and *parC* of *Escherichia coli*.

Gene	Genbank Accession no.	Forward primer (5'-3')	5' binding site	Reverse primer (5'-3')	5' binding site
gyrA	X06744	GCCATGAACGTACTAGGCAAT	158	AGAGTCGCCGTCGATAGAAC	348
parC	M58408	ATGTCTGAACTGGGCCTGA	145	TATTTCGACAACCGGGATTC	383

Table B. Forward and reverse primers for *gyrA* and *grlA* of *Staphylococcus aureus*.

Gene	GenBank Accession no.	Forward primer (5'-3')	5' binding site	Reverse primer (5'-3')	5' binding site
gyrA	M86227	CAAGGTATGACACCGGATA	166	TCCTCCATTAACTCAGCAA	635
grlA	L25288	CAGATGTTCGTGATGGTTT	95	TACCATTGGTTCGAGTGTC	465

Table C: Forward and reverse primers for *acrA, acrB* and 16S*rRNA* of *Escherichia coli.*

Gene	Genbank Accession no.	Forward primer (5'-3')	5' binding site	Reverse primer (5'-3')	5' binding site
acrA	ECK0457	ATTGGTAAGTCGAACGTGACG	550	AACTTAATGCCGTCACTGGTG	761
acrB	ECK0456	GATTACCATGCGTGCAACAC	1938	TCTGCAAGCAACTGGTTACG	2114
16S*rRNA*	EG30090	CAGCCACACTGGAACTGAGA	308	GTTAGCCGGTGCTTCTTCTG	511