The Mutant-Prevention Concentration (MPC):

Ideas for restricting the development of

fluoroquinolone resistance

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ABSTRACT

The mutant-prevention concentration (MPC) is a novel susceptibility measurement defined by a concentration threshold that would require cells to contain two concurrent resistance mutations for growth. Pneuococcal pneumonia, infections caused by *Pseudomonas aeruginosa*, and urinary tract infections caused by Gram-negative bacilli represent three distinct clinical situations for which fluoroquinolone-resistance occurs. MPC results were defined and measured for fluoroquinolones against clinical isolates of *Citrobacter freundii, Enterobacter cloacae, Escherichia. coli, Klebsiella pneumoniae, P. aeruginosa,* and *Streptococus pneumoniae.* Against clinical isolates of *S. pneumoniae,* MPC results for six fluoroquinolones were measured. Based on their potential for restricting the selection of resistant mutants, the six fluoroquinolones, in descending order, were found to be gemifloxacin > moxifloxacin > trovafloxacin > gatifloxacin > grepafloxacin > levofloxacin. For several compounds, 90% of clinical isolates that lacked a known resistance mutation had a MPC value that was close to or below the serum levels that could be attained with a dosing regimen recommended by the manufacturers. These data identify gemifloxacin, moxifloxacin and gatifloxacin as good candidates for determining whether MPC can be used as a guide for choosing and eventually administering fluoroquinolones to significantly reduce the development of fluoroquinolone –resistant *S. pneumoniae.* MPC_{90} results for 155 clinical isolates of *P. aeruginosa* against ciprofloxacin and levofloxacin were 4 and 16 μg/ml, respectively. Serum drug concentrations reported

previously for standard doses were above MPC_{90} for 5.5 hr for ciprofloxacin and 0 hr for levofloxacin. These data suggest that superior clinical performance of ciprofloxacin correlates with activity against resistant mutant subpopulations measured *in vitro*. MPC results were compared with minimum inhibitory concentrations (MIC) measurements preformed by agar dilution, and microbroth dilution and minimal inhibitory concentrations (MBC) for 100 clinical isolates of *C. freundii* (n=20), *E. cloacae* (n=20), *E. coli* (n=20), *K. pneumoniae* (n=20), and *P. aeruginosa* (n=20) for ciprofloxacin, levofloxacin and garenoxacin. MPC results were 2-to-8 fold higher than MIC or MBC results. Ciprofloxacin MPC results for *E.coli*, *C. freundii*, *E. cloacae*, *K. pneumoniae*, and *P. aeruginosa* were 0.5, 2, 1, 1, and 4 μg/ml, respectively. Levofloxacin, MPC results were were 1, 2, 4, 1, and 16 μg/ml, respectively. Garenoxacin, MPC were 1, 8, >8, 4, and >32 μg/ml, respectively. Garenoxacin had the highest MIC and MPC results and was the least active compound tested against isolates of *C. freundii*, *E. cloacae*, and *P. aeruginosa.* These data support the rational use of quinolones in the treatments of urinary tract infections and suppression of resistance. Incorporation of the MPC measurement into dosing strategies may preserve the longevity of antimicrobial compounds for future infectious diseases.

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ABBREVIATIONS USED

ATTC	American Type Culture Collection
AUC	Area under the concentration time curve
AUIC	Area under the inhibitory concentration time curve AUIC = AUC/MIC
AUC_{24}	Area under the concentration time curve during a 24 hr dosing interval
$AUIC_{24}$	Area under the inhibitory concentration time curve during a 24 hr dosing interval
BHI	brain heart infusion
CCCP	Carbonyl yanide *m*-chlorophenylhydrazone
CFU	Colony forming units
C_{max}	Maximum serum/tissue concentration
CHEF	Contoured clamped homogeneous electric field
CSB	Cell suspension buffer
EDTA	etheylenediaminetetracetic acid
EtBr	Ethidium bromide
FDA	Food and Drug Administration
GABA	Gamma amino buteric acid
I.P.	Intraperitoneal
I.V.	Intravenous
Kb	Kilobases
LMP	Low melting point
MDR	Multi-drug resistance
MHB	Mueller Hinton Broth
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
MPC	Mutant-prevention concentration
MPC_{pr}	Provisional Mutant-prevention concentration
MSW	Mutant-selection Window
n	Sample number
NCBI	National Center for Biotechnology
OMP	Outer membrane protein
PBR	Penicillin binding protein
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
PK/PD	Pharmacokinetic/pharmacodynamic
PMSF	Phenylmethyl-sulfonyl fluoride

qh8	Administration of an antimicrobial every 8 hours within a defined 24 hour interval
qh4	Administration of an antimicrobial every 4 hours within a defined 24 hour interval
QRDR	Quinolone Resistance Determining Region
R	resistant level of drug susceptibility
S	susceptible level of drug susceptibility
t	Time
TBE	Tris-HCl boric acid EDTA buffer
TE	Tris-HCl EDTA buffer
Temp	Temperature
THB	Todd-Hewitt Broth
T.I.D.	Three time daily dosing
TMP-SXT	Trimethoprim/sulfamethoxazole
TSA	Trypic soy agar
U	Units
UTI	Urinary tract infection

1.0 INTRODUCTION

1.1 Quinolone Antibiotics

The fluoroquinolones represent a relatively new class of broad-spectrum, systemically active antibacterial agents. Since the introduction of nalidixic acid in 1967 (304), fluoroquinolone usage has expanded far beyond an early role in the treatment of urinary tract infections (UTIs) and fluoroquinolones are now used in front-line therapies for the treatment of a number of different bacterial infections present at different anatomical sites. Collectively, the fluoroquinolone spectrum of activity now includes Gram-negative, Gram-positive, atypical and anaerobic pathogens (28, 33, 69), as well as multi-drug resistant organisms (MDR) (17, 164, 165, 246, 282), since the mechanism of quinolone action is distinct from existing classes of antimicrobials (165). Examination of many new derivatives has improved our understanding of quinolone activity and has led to structural changes in quinolone chemistry that in turn have produced compounds with longer elimination half lives, better dosing profiles, more extensive bacterial coverage, greater potency and acceptable safety profiles (30, 68, 96, 113, 132). Overall, the introduction of fluoroquinolones into clinical practice, particularly as orally administered antibiotics, has revolutionized the management of a number of infections that were previously treatable only with parenteral antimicrobial agents. Thus, preserving the current and future clinical utility of fluoroquinolones is of paramount importance. The following chapter discusses quinolone history, structure/activity relationships, and development of resistance and introduces a novel susceptibility

parameter, termed, mutant-prevention concentration (MPC) which is designed to minimize fluoroquinolone resistance while maximizing therapeutic outcome.

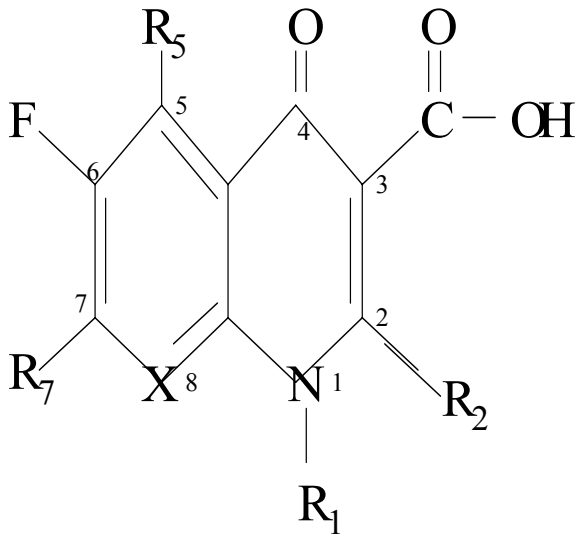
1.2 History and Development of Quinolone Agents: Structure - Activity Relationships

The discovery of quinolone antibiotics began by chance during the early 1960s when a by-product of the commercial preparation of the antimalarial drug chloroquine was found to have antibacterial properties (201). The compound 7-chloro-1-ethyl-1, 4-dihydro-7 methyl-4-oxo-1, 8-napthyrlidin-3-carboxylic acid was subsequently modified with the additions of N-1 ethyl and C-1 methyl groups which resulted in nalidixic acid, the first quinolone antibiotic. Nalidixic acid was introduced for the treatment of uncomplicated UTIs in 1964 (132, 201, 304) and marked the beginning of four decades of quinolone use and development.

In addition to nalidixic acid, there are nine quinolone agents currently approved for use in Canada and in the United states, including ciprofloxacin, enoxacin, gatifloxacin, gemifloxacin, levofloxacin, lomefloxacin, moxifloxacin, norfloxacin, and ofloxacin (15, 16, 262). Additional members of the class, such as perfloxacin, fleroxacin and tosufloxacin, are in use outside North America (54, 187, 191). Gemifloxacin was recently approved for use in North America (2004) and is now in clinical practice in the U.S.A. and expected to be approved for use in Canada. Classification of the quinolones has not been officially formalized and various criteria, including spectrum of activity, chemical structure, clinical utility and decade of development have all been proposed as a means for grouping members of the

fluoroquinolone class of antimicrobials (12, 28, 132, 253, 272). For example, Ball (12) has proposed that first generation compounds, such as nalidixic acid and flemequin, provide coverage primarily for the Enterobacteriaceae. Second-generation compounds, such as ciprofloxacin, levofloxacin and sparfloxacin are further divided into those with enhanced, predominant Gram-negative coverage with balanced broad-spectrum activity including some Gram-positive bacteria. Third-generation agents, such as moxifloxacin and gatifloxacin, display enhanced Gram-positive activity, particularly against *Streptococcus pneumoniae* (12, 30). Gootz *et al* (132) proposed a classification of quinolones based on incorporating key modifications in the chemical structure that correlate with the decade of development. Despite these distinctions, quinolone chemistry and activity are inextricably linked and quinolones generally fall into one of three distinct groups consisting of first, second, or third-generation agents (9, 28, 30, 81, 132). All currently approved quinolones share a common core structure with that of the original quinolone, nalidixic acid, consisting of two fused 6-member heterocyclic nuclei containing one to four nitrogen atoms (12, 81, 132) (Figure 1.1.).

Figure 1.1 Chemical Structure of the Quinolone Nucleus



Side chains present at positions N-1 and R-1 have been shown to control theophylline interactions and genetic toxicity. An R-5 moiety influences phototoxicity and genetic toxicity. A fluorine atom at the C-6 carbon position has been associated with increased potency; all currently available "fluoroquinolones" retain a 6-fluorine atom. Side chains at the C-7 postion has been associated with gamma amino buteric acid (GABA) binding and theophylline interactions. C-8 rings have been associated with enhanced killing and increased activity against *S. pneumoniae.*

Nalidixic acid was the first clinically useful quinolone that possessed excellent *in vitro* activity against a variety of different Gram-negative species from the *Enterobacteriaceae* (304, 329). However, it proved ineffective against various genera and species of Gram-positive bacteria, *Pseudomonas aeruginosa* and *Serratia marcescens* (10, 73, 107, 233), and never became a useful agent in the treatment of systemic infections because it possessed poor pharmacological properties. Other early compounds such as rosoxacin, oxolinic acid and cinoxacin followed, however, none of these compounds significantly improved upon the activity or clinical utility of earlier generation agents and, as a result, did not obtain secure status within the quinolone class (227). Overall, first generation agents are characterized by their Gram-negative coverage (excluding *P. aeruginosa*). However, resistance among Gram-negative bacteria was quick to develop and first generation agents possessed low adsorption and tissue penetration ($\leq$ 0.5 mg/ml peak serum concentrations) which relegated their use to infections of the urinary tract (173, 304).

Development in the 1970s focused on producing more potent second-generation agents and two significant breakthroughs in the evolution of the quinolone class occurred with the additions of a piprazynl ring, or piperazine derivative, at the C-7 position and a fluorine atom on the C-6 carbon. Addition of a 7-piperazine ring created pipemidic acid, a quinolone with enhanced penetration of the bacterial cell wall which improved activity against Gram-negative bacteria and some Gram-positive bacteria (12, 326). The incorporation of a fluorine atom at position C-6 resulted in flemequine. The

C-6 fluorine was shown to enhance both gyrase inhibition and bacterial cell penetration (82), improving activity against Gram-negative and Gram-positive organisms. The high degree of intrinsic activity associated with compounds possessing a C-6 fluorine has led to acceptance of the fluorine atom in overall "fluoroquinolone" chemistry (103, 273). Subsequently, all currently approved quinolones retain a fluorine atom at position C-6.

In 1980, Koga *et al* (188) discovered that inclusion of a C-7-piperazinyl ring in the quinolone nucluess further increased the spectrum of activity of the quinolones (185). The exchange of a carbon molecule for nitrogen at position 8, coupled with the C-6 fluorine atom and a C-7 ring, resulted in norfloxacin-the first of the modern day fluoroquinolones. Norfloxacin was approved for clinical use in 1984 (103, 268) and was characterized as having high activity (and improved activity) against various genus and species of *Aeromonas hydrophila, Haemophilus influenzae, P. aeruginosa, Pasteurella multocida, Neisseria* spp. and (228). The introduction of norfloxacin signified the beginning of a period of rapid development of other oral second generation quinolones such as ciprofloxacin, enoxacin, fleroxacin, ofloxacin, and perfloxacin. Of these, only ciprofloxacin and ofloxacin became widely established for the treatment of infections involving sites other than the urinary tract (12).

Ciprofloxacin, released in 1987, incorporated the addition of an N-1 cyclopropyl group and was characterized as having improved activity against Gram-positive organisms such as *Enterococcus faecalis, Staphylococcus aureus, S. pneumoniae,* and against Gram-negative organisms such as *Escherichia coli*, *H. influenzae, K.*

pneumoniae and *Neisseria gonorrhoeae.* Ciprofloxacin was also active against atypical organisms such as *Chlamydia* spp., *Legionella* spp., and *Mycoplasma pneumoniae,* showed good oral bioavailability (20, 163, 311, 356). Ciprofloxacin marked two important milestones in the development of fluoroquinolones. It was the first available intravenous (I.V.) agent allowing for sequential or step down from intravenous to oral therapy in hospitalized patients with serious systemic infections. This proved to be a significant cost containment benefit (311). It was also first the first orally available antimicrobial agent with reliable activity against *P. aeruginosa* at sites other than the urinary tract (36, 39, 51, 72, 167). Ofloxacin, a chiral fluorinated 1,8-cylco compound developed in the mid 1980s, also had broad spectrum applications and was approved for use in 1990 (338). In comparison with ciprofloxacin, ofloxacin is rapidly absorbed from the gastrointestinal tract, achieves higher serum concentrations and has a longer elimination half-life (28, 202, 312, 313). *Enterobacteriaceae*, enteropathogens (bacterial) and fastidious Gram-negative bacteria are highly susceptible to ofloxacin which display minimal inhibitory concentrations (MICs) of ≤ 2 μg/ml. Ofloxacin MICs against *P. aeruginosa* are generally 2-to-8 fold higher than those observed for ciprofloxacin (341). Levofloxacin, a racemic mixture of the active component of ofloxacin present in the L-isomer form was released shortly after ofloxacin and approved for use in United States in 1999 (269).

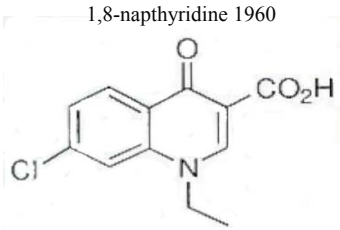
Levofloxacin expanded upon the Gram-negative coverage of ofloxacin and is generally considered to be 2-fold more potent than ofloxacin based on lower MICs (72,

170). Levofloxacin possesses Gram-positive coverage against *S. aureus,* borderline (near-breakpoint) activity against *S. pneumoniae* and became the first quinolone approved for once daily therapy of community-acquired pneumoniae in North America**.** Molecular substitutions based on the 6-fluorine, 7-piperazinyl molecule yielded additional second-generation agents such as sparfloxacin and clinafloxacin which demonstrated increased activity against *S. aureus* and *S. pneumoniae* relative to earlier generation agents, but which for a variety of reasons did not reach wide-spread clinical use. For example, the pharmacokinetics of clinafloxacin were such that it necessitated twice or three times daily dosing (132, 297, 298)**.** Collectively, norfloxacin, ciprofloxacin, ofloxacin/levofloxacin, sparfloxacin and clinafloxacn represent second-generation agents or extended spectrum quinolones, which were introduced into clinical practice in the late 1980s and 1990s. The primary advantage of second-generation agents is an improved spectrum that includes *H. influenzae, Moraxella catarrhalis, Neisseria* spp., *P. aeruginosa*, and, *Staphylococcus* spp. and atypical pathogens such as *Chlamydia* spp., *Legionella* spp., and *Mycoplasma* spp.

The search for broader-spectrum fluoroquinolones with greater potency against Gram-positive and anaerobic bacteria fuelled the development of quinolones in the mid to late 1990s. A large number of the investigational compounds possessed excellent activity against gram-positive organisms, but frequently lost potency against Gram-negative organisms. However, a small number of derivatives were discovered that had improved activity against Gram-positive bacteria and also retained good anti-Gram-

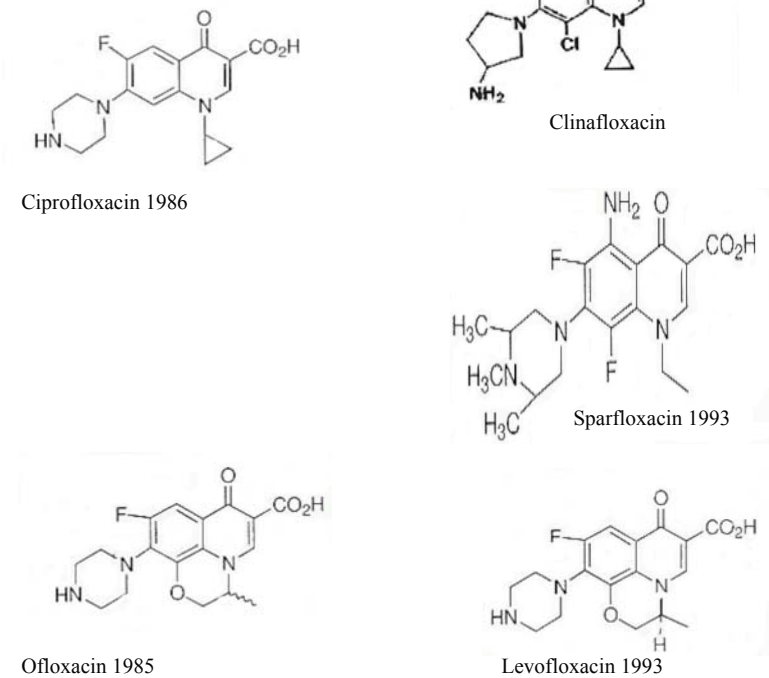
negative properties. These newer derivatives represent third-generation quinolones, or "respiratory quinolones". Third-generation agents contain variations of C-7 rings (7-3-methyl-piperazinyl in the case of gatifloxacin and a 7-azabicyclo ring in the case of moxifloxacin) and improved activity against *S. pneumoniae* and anaerobic bacteria. Currently, all approved third generation agents also posses C-8 methoxy residues which are associated with increased bacterial killing (221, 368). Trovafloxacin, grepafloxacin and garenoxacin are no longer considered clinical alternatives, but are noteworthy in the overall development of third-generation agents because structural modifications to C-3, C-5 and C-7 carbon atoms have led to increased understanding of the structure-activity relationship of fluoroquinolones (35, 59-62, 171). Garenoxacin can be further distinguished from other fluoroquinolones because it was the first quinolone lacking a fluorine atom at the C-6 position to advance to stage III clinical trials (120). Figure 1.2 outlines the development of selected fluoroquinolones.

Figure 1.2 Evolution of Quinolone Development



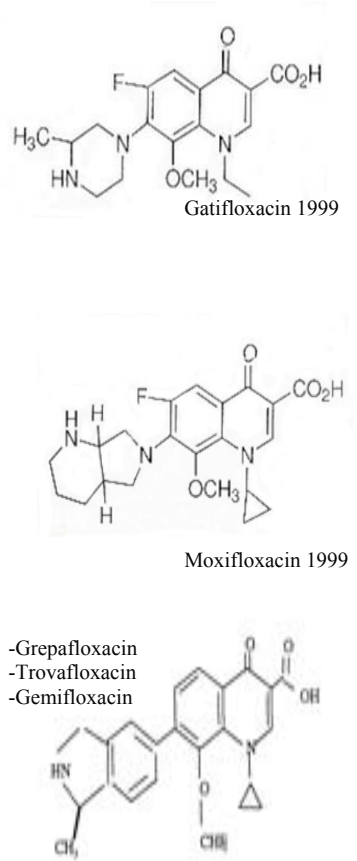
Enterobacteriaceae

1st GENERATION



Enterobacteriaceae + P.aeruginosa + S. aureus + M. catarrhalis + N. gonorrhoeae + S. pneumonaie

2nd GENERATION



S. pneumoniae + S. aureus + anaerobes

3rd GENERATION

Selected quinolone agents highlight developments in quinolone antimicrobial agents. Quinolones are grouped by first, second, or third-generation based on the combination of decade of development and spectrum of activity.

1.3 Intracellular Quinolone Targets and Quinolone Action

Quinolones interact with two related, but distinct bacterial topoisomerase enzymes. Bacterial topoisomerases are a class of enzymes essential in maintaining a chemically stable and biologically active form of cellular DNA (169). There are four bacterial topoisomerases, classified as either type I or type II enzymes. Type I topoisomerases are active during the replication of single-strand DNA, whereas type II topoisomerases are required for double-stranded DNA replication (204, 324). Quinolone antibiotics are strong inhibitors of the type II enzymes, which include DNA gyrase (topoisomerase type II) and topoisomerase type IV. Type I enzymes are not sensitive to the inhibitory activity of quinolones (244). DNA gyrase and Topoisomerase IV exist as a tetrameric protein, each consisting of two subunit dimers. GyrA and gyrB comprise DNA gyrase and are encoded by the *gyrA* and *gyrB* genes, while ParC (GrlA in *S. aureus*) and ParE (GrlB in *S. aureus*) are encoded by the *parC* and *parE* genes, and comprise topoisomerase IV. DNA gyrase and topoisomerase IV facilitate bacterial replication by breaking both strands of duplex DNA, passing another strand of DNA through the break and resealing initial broken strands (122, 165, 350). The activity of g*yrA* and p*arC* subunits are responsible for cutting double stranded DNA utilizing the free energy of ATP hydrolysed by the *gyrB* or *parE* subunits (18). Once the DNA is cut, another strand of DNA is passed through the gap and the DNA is

re-ligated. DNA gyrase differs from topoisomerase IV in that it can wrap DNA around itself and cause strand passage of the molecule of DNA that it has cut. In the case of topoisomerase IV, strand passage occurs during a process which utilizes two different DNA molecules. This process occurs in such a way that after each catalytic event, the linear DNA double helix becomes twisted, resulting in a superhelix structure. This superhelical twisting is referred to as negative supercoiling and occurs in the opposite direction to the right-handed coiling of the internal helix of the DNA strand. Negative supercoiling, induced by the actions of DNA gyrase, allows chromosomal DNA to become tightly packaged within the bacterial cell and ensures the correct conformation for the initiation of DNA replication (88, 166). DNA gyrase also allows the continuation of DNA replication by producing negative supercoils in front of the DNA replication fork, thereby counteracting the positive supercoiling introduced by the action of DNA replication (223, 349). The bi-directional nature of the DNA replication process is such that daughter molecules are interlinked, or catenated, after replication. Topoisomerase IV is the principal enzyme that decatenates or removes the interlinking of daughter chromosomes at the completion of each round of DNA replication, thus allowing their segregation into daughter cells (361).

1.4 Mechanisms of Quinolone Action

During replication, bacterial DNA interacts with type II topoisomerase enzymes, resulting in a DNA-enzyme cleavage complex, which becomes the substrate for quinolone binding (153). Interaction of quinolones with DNA-enzyme complexes (DNA with DNA gyrase or topoisomerase IV) results in conformational changes to both enzyme and enzyme-bound DNA (180, 193, 228, 229). Topoisomerase enzymes break DNA strands and the interaction of the quinolone-enzyme-DNA complex prevents re-ligation of the broken DNA strands (123, 151, 331). Although not completely understood, quinolone action involves a two-step process beginning with the stabilization of quinolone-enzyme-DNA complexes leading to trapping of double-stranded DNA breaks and the subsequent release of double-stranded DNA breaks (88). Although quinolone-enzyme-DNA complex formation and cell death are clearly related, the bacteriostatic and bactericidal actions of quinolones action appear to represent distinct events. For example, the formation of quinolone-enzyme-DNA complexes cannot result in cell death because complex formation, as monitored by inhibition of DNA synthesis, is reversible (134, 155). Analysis of closely related fluoroquinolones has demonstrated that a compound can be more effective at preventing colony formation, but less effective at killing cells (131, 245, 369). A generalized model of the bactericidal action of quinolone antibiotics proposes that drug-enzyme-DNA complexes are sufficient in blocking cell growth, while the release of DNA breaks from drug-enzyme-DNA complexes is the lethal event. This assumption correlates well with *in*

vitro findings demonstrating that quinolone concentrations required to release double-stranded breaks from chromosomal drug-enzyme-DNA complexes correlate better with cell death than with inhibition of growth (52, 90). Therefore, quinolones that trap pairs of single-stranded breaks, or more readily cause release of pairs of single-stranded breaks, are expected to be more lethal.

1.5 Mechanisms of Quinolone Resistance

The most common mechanisms of quinolone resistance occur via mutations within the target genes *gyrA/gyrB* and *parC/parE*. Resistance to fluoroquinolones develops in a step-wise fashion, with incremental rises in quinolone MICs at each stage (281). Reductions in susceptibilities are associated with initial, first-step mutations that occur at specific sites within *gyrA* and *parC* genes, known as quinolone-resistance-determining regions (QRDR) (28, 88, 165, 166). Subsequent second-step mutations can occur in QRDR of the *gyrB* and *parE* genes which lead to high-level resistance. A common model of quinolone target modifications proposes that amino acid changes in the QRDR of DNA gyrase and topoisomerase IV alter the structure of the quinolone binding site near the interface of the enzyme and DNA; subsequent resistance is then an effect of reduced drug affinity for the modified enzyme-DNA complex (166). However, direct structural information on the site of quinolone-binding within the complex is not yet available.

The affinity of a quinolone against any bacterial pathogen appears to result from the relative sensitivities of DNA gyrase and topoisomerase IV. The more sensitive

enzyme generally determines the primary quinolone target for a given organism, independent of the sensitivity of the secondary target. For Gram-negative bacteria, purified DNA gyrase is more sensitive to quinolones than purified topoisomerase IV, leading to the hypothesis that DNA gyrase is the primary quinolone target among Gram-negative bacteria. Initial genetic studies with nalidixic acid-resistant mutants of *E. coli* demonstrated that first-step mutations occurred in a region of the GyrA, between amino acid sites 67-106 (123, 140, 255, 360). Resistance mutations in *gyrB* of Gram-negative bacteria develop as secondary mutations to those of *gyrA* and are associated with highly resistant organisms (166). Conversely, in *S. aureus* purified topoisomerase IV is more sensitive to quinolone action than DNA gyrase (24, 165). Proof that topoisomerase IV is a primary quinolone target among Gram-positive bacteria came from studies in which first-step quinolone resistance mutations were found in regions of the *parC (grlA)* genes for clinical isolates of *S. aureus* and *S. pneumoniae* (104, 105, 260). Thus, primary target affinity for Gram-negative organisms appears to be DNA gyrase while topoisomerase IV is the primary target in Gram-positive bacteria.

However, this simplified stratification of quinolone target affinity is incomplete when examining newer third-generation agents such as moxifloxacin and gemifloxacin which have been shown to target DNA gyrase in Gram-positive organisms while retaining affinity for topoisomerase IV. For example, in *S. pneumoniae*, sparfloxacin and gatifloxacin selected first step *gyrA* mutants (117, 278), and purified *S. pneumoniae* DNA gyrase was found to be less sensitive to sparfloxacin and clinafloxacin than

purified topoisomerase IV (246, 277). The explanation for the apparently anomalous behaviour of sparfloxacin, gatifloxacin and clinafloxacin is yet unclear, but has prompted the argument that quinolone chemistry may determine target affinity (81, 88). As such, compounds may become classified into one of three archetypal mechanistic classes (32, 152, 276, 278): (1) compounds which select gyrase mutations before topoisomerase IV mutations and likely act through DNA gyrase *in vivo*, (2) compounds that select for QRDR mutations in the genes encoding topoisomerase IV first before those in DNA gyrase, thereby suggesting that the drugs act preferentially through topoisomerase IV *in vivo* and (3) compounds which posses dual target activity and act through both DNA gyrase and topoisomerase IV. Fluoroquinolones with comparable target affinity (i.e., both DNA gyrase and topoisomerase IV) have changed the perception of how resistance mutations are thought to accumulate in target genes. For resistance mutations in the primary target enzyme, the level of sensitivity of the unmutated secondary target enzyme, which becomes the more sensitive enzyme when the primary target is resistant, may determine the degree of resistance. This hypothesis implies that for different quinolones, the level of resistance conferred by a mutation in the primary target enzyme would decrease as the level of drug sensitivity of the secondary target approaches that of the primary target. Furthermore, it implies that concurrent mutations in both target enzymes would be required for resistance due to target alterations for any quinolone that had equal potency against DNA gyrase and topoisomerase IV. This suggestion appears to be the case with *S. pneumoniae* and

clinafloxacin (277). Mutants of *S. pneumoniae* selected with clinafloxacin occur at a low frequency and *gyrA* mutations selected in first-step experiments had a minimal impact on clinafloxacin resistance. Second and third-step mutants of *gyrA* and *parC*, however, exhibit substantial resistance (165, 276, 278).

1.6 Reduced Intracellular Concentration

In order to reach their targets in the cell cytoplasm, fluoroquinolones must cross the cytoplasmic membrane and, in Gram-negative bacteria, the outer membrane as well. Thus, alterations in drug permeation contribute to overall decreases in quinolone susceptibility. In addition to passive membrane diffusion, the relative size and charge of fluoroquinolones facilitate active transport across the outer membrane through porin proteins which form general diffusion channels allowing access to the cell (132, 164, 266). Quinolone-resistant clinical isolates of Gram-negative bacteria have been found to posses reduced numbers of outer membrane porins. For example, deficiencies in the outer membrane proteins (OMP) OmpF and D2 of *E.coli* and *P. aeruginosa* have been associated with resistance to a fluoroquinolones (237, 242). Reductions in the relative amount of OMPs result in decreased diffusion of quinolones across outer membranes (157, 158, 288), however, examination of diffusion rates suggest that porin reductions alone are generally not sufficient to account for high-level resistance (266).

Recently, resistance caused by reduced accumulation has been shown to result from enhanced expression of efflux systems that actively pump drug from the cytoplasm. In Gram-negative bacteria, these systems typically have three components:

the efflux pump located in the cytoplasmic membrane, an OMP and a membrane fusion (or linker) protein (164, 291). Efflux systems actively extrude drug from the cytoplasm or cytoplasmic membrane across the periplasm and outer membrane to the cell exterior. The energy for this process is derived from the proton gradient across the membranes. Efflux expression is regulated and resistance occurs by chromosomal mutations that causes coordinated increased expression of pump components (164). The conditions that contribute to increased expression of efflux system components remain largely unknown. Efflux mechanisms that contribute to MDR, including resistance to fluoroquinolones, have been identified in many clinical isolates. These include: *Bacillus subtilis, Citrobacter spp., E. coli, Enterobacter spp., H. influenzae, K. pneumoniae, Mycobacterium spp., N. gonorrhoeae, P. aeruginosa, Salmonella typhimurium, S. aureus, and S. pneumoniae* (263, 308). Chemical structures of various fluoroquinolone may determine the extent to which efflux systems operate in specific bacterial species. The correlation between chemical structure and efflux activity have not been fully defined, but appear to correlate with the relative degree of hydrophilicity for various compounds (63, 354, 360).

1.7 Key Pharmacokinetic and Pharmacodynamic Factors for Fluoroquinolone Antibioitcs

Many methods have been used to evaluate *in vitro* activities of antibacterial agents. Most commonly, MIC and minimal bactericidal concentrations (MBC) measurements are used to assess antibacterial potency as assessed by inhibition or killing of a pathogen at an endpoint of 18-24 hr. Determination of MIC/MBC endpoints

following the incubation of an organism in the presence of a constant antibiotic concentration reflects a static measurement of the antibiotic's bacteriostatic/bactericidal activity (68). As a result, MIC/MBC measurements do not provide data on the time-course of antimicrobial action, including the duration of drug exposure required for bacterial eradication, the rate of bactericidal activity or persistent effects of the antimicrobial agents (65, 68, 364). Thus, the selection of optimal fluoroquinolone dosing regimens requires careful consideration of microbiological responses to an infecting organism (pharmacokinetics (PK)) and patient-specific factors related to drug exposure, most notably toxicity (pharmacodynamics (PD) (65). Understanding the PK/PD relationship of fluoroquinolone antibiotics can facilitate selection of optimal dosing regimes which serve to hasten the antimicrobial response to an invading pathogen, prevent treatment failures, minimize the development of resistance and maintain the therapeutic lifespan of the antimicrobial agent. Therefore, experimental models that reflect PK/PD dynamics for specific quinolones against specific pathogens present at specific anatomical sites should provide additional clinically meaningful information about the potential of an antibiotic.

Generally, antibacterial effects correlate with one of three pharmacokinetic parameters:

1. The ratio of peak drug concentration (C_{max}) of an agent to its MIC for the bacterium (C_{max}/MIC), termed concentration dependent.

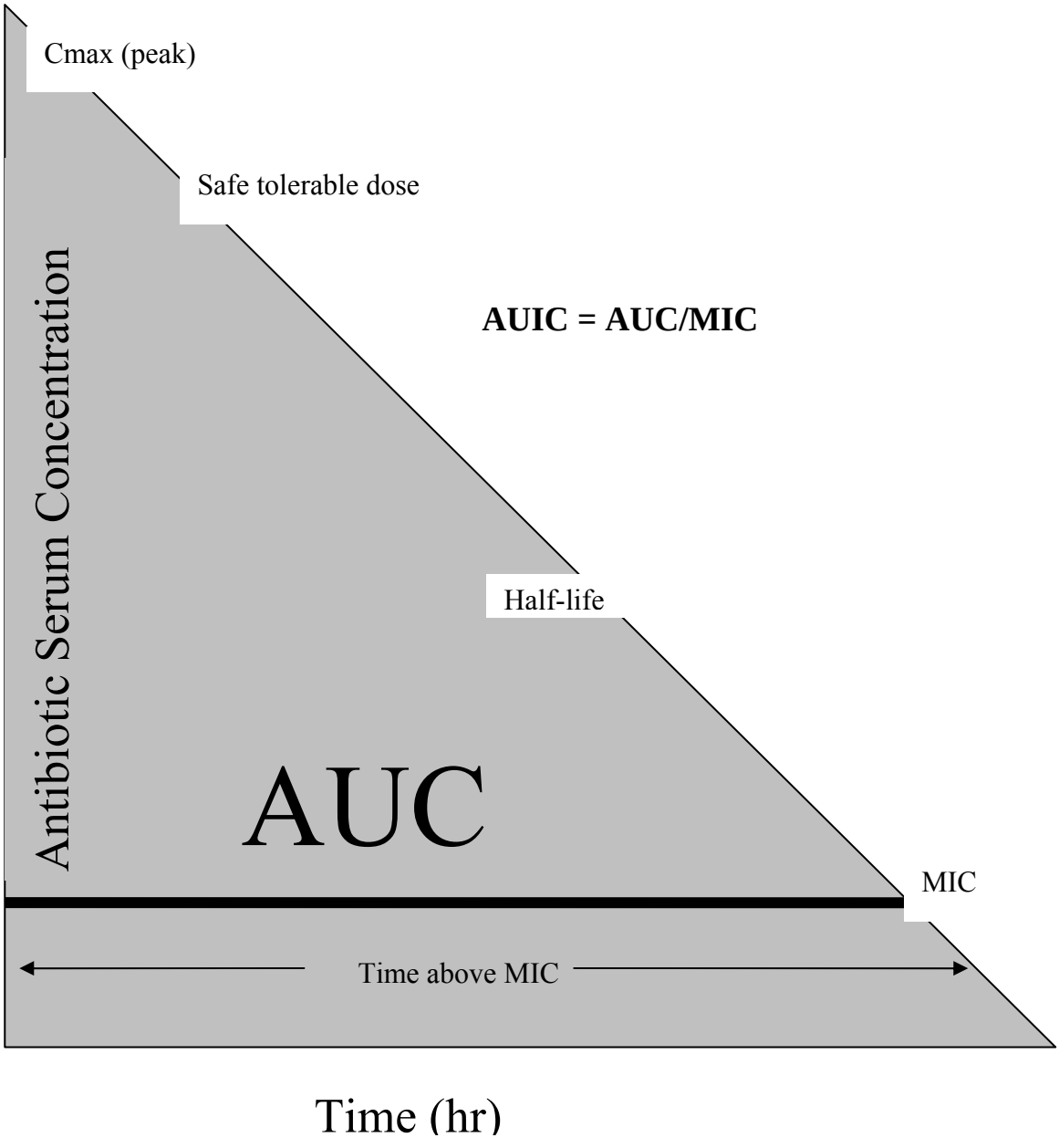
2. Time (T) of exposure of a bacterium to serum drug concentrations exceeding the MIC (T>MIC), termed time dependent.
3. The ratio of the area under the drug concentration versus-time curve (AUC).

On the basis of the PK principle dictating their action, antibiotics are typically characterized as either concentration-dependent or time-dependent agents (68, 318). For example, the time above the MIC is an important parameter used with β-lactam compounds because bacterial inhibition and kill rates at high concentrations are virtually identical to rates at concentrations near the middle of a concentration time curve, or even at the MIC drug concentration (92). Thus, for β-lactams, the most important principle in bacterial eradication is not increasing concentration, but the length of time achieved above a defined drug concentration, usually the MIC. By comparison, for concentration-dependent antimicrobial agents, the kill rate at concentrations near the peak is greater than that at concentrations near the middle of the curve (91). Fluoroquinolones exhibit concentration-dependent responses which traditionally have been described by measuring (C_{max}) in relation to the MIC (C_{max}/MIC) (93, 94, 321). C_{max}/MIC ratios of 8-to-10 correlate with increased clinical success and reduced likelihood of selecting resistant strains (318).

The AUC represents the newest pharmacological measure which is used to predict the therapeutic response of microorganisms to antimicrobials. The AUC correlates drug exposure with a defined dosing interval which is then reported as the area under the plasma drug concentration time curve (320, 321). The AUC is an

empirically derived formula based on the MIC measure of drug potency. For example, upon introduction of an antibiotic into tissue/serum, the drug accumulates and achieves (C_{max}), which is dictated by both the properties of the antibiotic and the infected anatomical site. As the concentration declines over time, it intersects with of the MIC of a particular pathogen. The area created between the C_{max} and MIC as drug levels decline is represented by a numerical value defined as the AUC. The AUC is normally calculated over 24 hr and is designated as the AUC_{24}. The ratio of the AUC/MIC defines the area under the inhibitory concentration time curve (AUIC) (Figure 1.3). A landmark study by Forrest *et al* (115) reported that an optimal AUC_{24}/MIC ($AUIC_{24}$) for ciprofloxacin of $\geq$125 was associated with bacterial eradication in critically ill patients with lower respiratory tract infections caused by Gram-negative bacilli. Reduced drug exposure and low AUIC ratios correlate directly with emergence of resistance and ratios below 100 have correlated with a 50-83% probability that resistance will develop (133, 318, 336). Therefore, for fluoroquinolones, an AUIC of at least 125 (serum inhibitory $titre^{-1}$) should be targeted, because values <100 have been associated with the development of resistance (115, 133, 317).

Figure 1.3 The Area Under the Concentration Time Curve (AUC)



The AUC is an empirically derived formula used to predict clinical success and development of resistance for fluoroquinolones based on the relationship between clinical pharmacokinetics and the MIC of a particular pathogen. Upon administration of an antimicrobial, the antibiotic concentrates and eventually reaches the Cmax drug concentration which typically falls within a safe and tolerable dose for the patient. As the concentration declines over time, it eventually intersects with the MIC of the pathogen. The half-life of the antimicrobial agent represents the time it takes for drug concentrations to achieve half the Cmax for a given dose. The Area created between the Cmax and the MIC for a given drug-organism combination defines the AUC. AUC values $\geq$ 125 and C_{max} /MIC ratios of 8-to-10 correlate with *in vivo* success for fluoroquinolones against Gram-positive and Gram-negative organisms.

been found to be associated with clinical efficacy (11, 198, 213, 214, 224) and has prompted the question of whether AUIC values $\geq$125 need to be achieved against Gram-positive pathogens (91, 249). In a clinical study with levofloxacin and *S. pneumoniae,* clinical success was observed in patients where AUIC values $\leq$ 50 were documented (295) and acceptance of lower AUIC values of 30-to-50 was proposed on the premise that successful clinical outcomes would occur even with low AUIC values (<125). However, 85% of patients had mean AUIC values >100 and it is likely that few patients enrolled in the study had AUIC values in the 30-to-50 range because organisms with MIC values of 2 μg/ml to levofloxacin were uncommon during the early 1990s. Currently, organisms with MICs of 1-2 μg/ml to levofloxacin are now being identified (31, 159) and these organisms would confer levofloxacin AUIC values of approximately 25.7 (318), thereby re-raising the argument that AUIC values of $\geq$100 should be targeted for Gram-positive organisms. Recently, an *in vitro* pharmacodynamic model, specifically designed to incorporate MPC measurements demonstrated that AUIC values of $\leq$100 were associated with decreases in *S. aureus* susceptibility to fluoroquinolones, while values $\geq$100 did not result in changes in MICs (371)

The question as to which ratio (i.e., C_{max}/MIC or AUC/MIC) is a better predictor of antimicrobial efficacy remains unresolved. Some studies have shown that AUC_{24}/MIC is the best predictor for success with quinolones (43, 224-226), while others have suggested that the Cmax/MIC is a better predictor of bacterial eradication

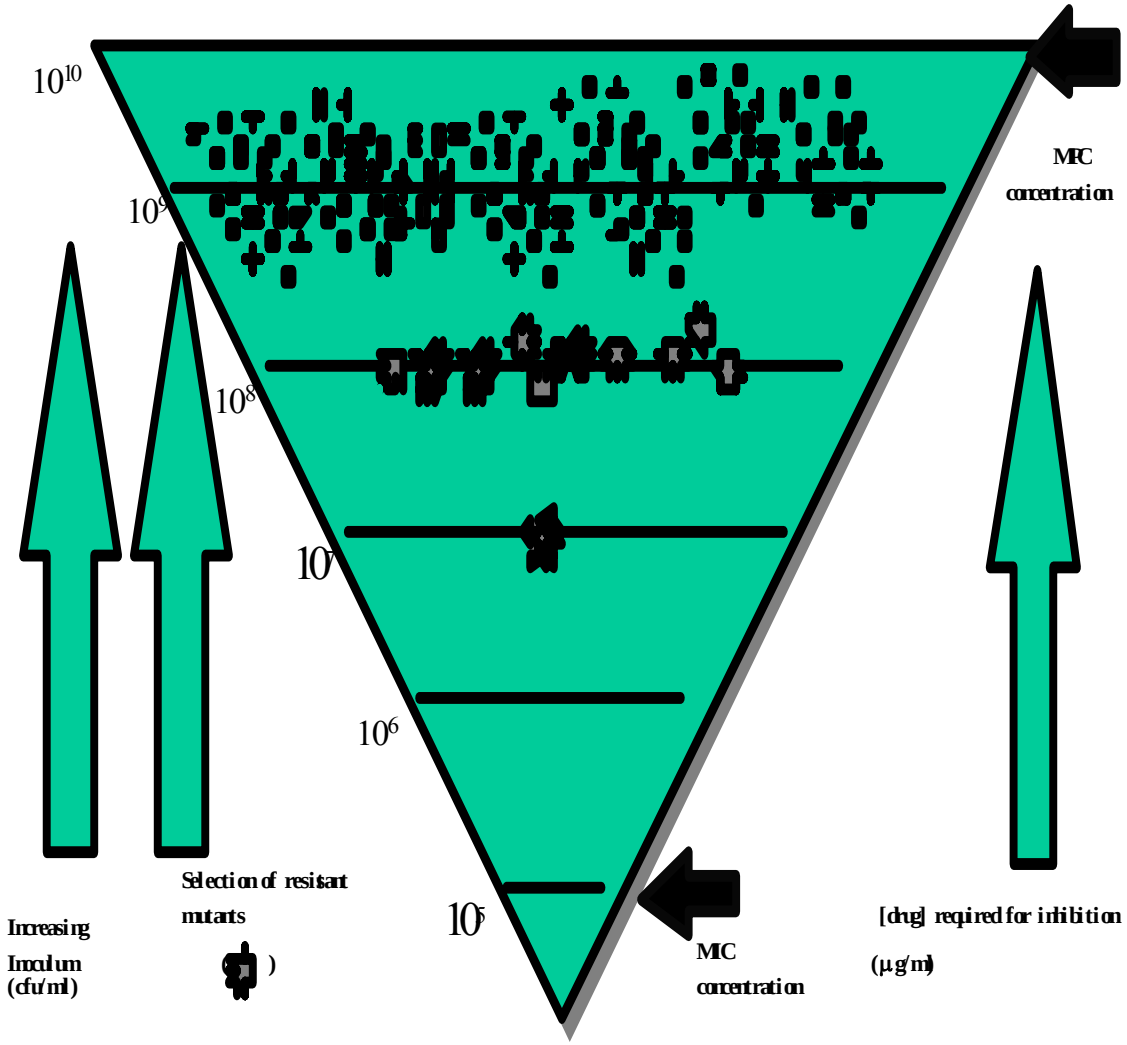
(25). Ultimately, the activity of an antimicrobial compound and the subsequent clinical success is dependent on the combination of specific binding to intracellular targets in the pathogen and occupation of the critical binding sites for a minimum period of time. Therefore, neither the C_{max} /MIC ratio nor the AUIC alone will predict a compound's antibacterial effect. Recently, it has been suggested that if a C_{max}/MIC ratio greater than 10 is achieved, C_{max}/MIC will be the most important parameter in determining outcomes (295), whereas at C_{max}/MIC ratios of <10, the AUC_{24}/MIC ratio is a better predictor of clinical outcome and decreased incidence of resistance (43, 226, 295, 318). Scaglione *et al* (314) rationalized these distinctions by examining the effect of protein binding on the relative contributions of AUC/MIC and C_{max}/MIC ratios to the overall fluoroquinolone effect. Based on their findings, the authors concluded that a larger fraction of free drug exists at peak concentrations than at lower concentrations and a larger fraction of free drug may have a greater effect because even with the same AUC, a higher peak concentration will result in a larger fraction of free drug. Thus, it is not the peak concentration itself that leads to a better outcome, but the fact that the AUC of the free fraction is larger if the peak concentration is higher (314).

1.8 The Mutant-Prevention Concentraton (MPC): *In vitro* Masurement of the MPC and Experimental Determination of the Mutant-Selection Window

Fluoroquinolone antibiotics are important antimicrobial compounds used to treat infections caused by Gram-negative and Gram-positive bacteria at a number of different anatomical sites. The use of newer more potent agents for front line therapy is advocated as the most prudent use of antibiotics (78). Today's modern-generation fluoroquinolones are highly active against Gram-positive organisms such as *S. aureus* and *S. pneumoniae,* but reports of treatment failures and documented *in vitro* resistance has raised concerns that resistance may develop to these new agents (7, 41, 53, 70, 89, 315, 371). The development of antibiotic resistance correlates with increased use. For example, since the late 1980s and early 1990s, ceftazadime, a third-generation cephalosporin, has been widely used against Gram-negative pathogens, *Citrobacter* spp., *E. coli, Enterobacter* spp., *K. pneumoniae,* and *P. aeruginosa.* The subsequent emergence of broad spectrum β-lactamase-producing Gram-negative bacteria has been attributed to indiscriminate use of third-generation cephalosporins (58, 236, 301, 302, 315). However, increases in consumption cannot fully account for changes in resistance patterns. Eliminating the misuse and overuse involved in antibiotic utilization will fail to correct resistance problems because traditional dosing strategies are only one mutational step ahead of the pathogen, a factor which will ultimately erode new agents, if and when they become available. Therefore, strategies specifically designed to minimize the development of resistance must accompany the development and use of antimicrobial agents.

It has been suggested that if bacterial cells must attain two concurrent resistance mutations for growth in the presence of a quinolone, then few mutants would be selectively amplified because double mutations should rarely occur (32, 41, 87, 142, 367, 369). Bacterial populations may reach 10^{10} cells in human infections, but at a mutation frequency of 10^{-7}, more than 10^{14} bacteria ($10^7 \times 10^7$) would be required to detect two concurrent fluoroquinolone-resistant mutations. Current susceptibility practices rely on standardized inoculums, based on the application of 10^5- 10^6 colony forming units (CFU), which is too low to account for the presence of first-step resistant subpopulations that are present at the sites of infections. Figure 1.4 illustrates the factors involved in the MPC measurement. When we examined the effect of fluoroquinolone concentration on the selection of resistant mutants of *Citrobacter freundii*, *Enterobacter cloacae, E. coli, K. pneumoniae, P. aeruginosa,* and *S. pneumoniae*, we found a concentration with each organism at which no mutant was recovered when 10^9 to 10^{10} cells were applied to agar plates (41, 143, 145, 148, 149). This drug concentration, which we designated as the MPC, would require a bacterial cell to develop more than one resistance mutation for growth. Thus at concentrations above the MPC, a bacterial population greater than that normally present during infection would be necessary to observe outgrowth of a resistant mutant. Since fluoroquinolone structure affects the value of the MPC (83), it appears that the MPC might serve as a simple measure of antibiotic potency that incorporates the ability of a compound to restrict selection of resistant mutants (41, 89, 149, 367).

Figure 1.4 MPC Dynamics



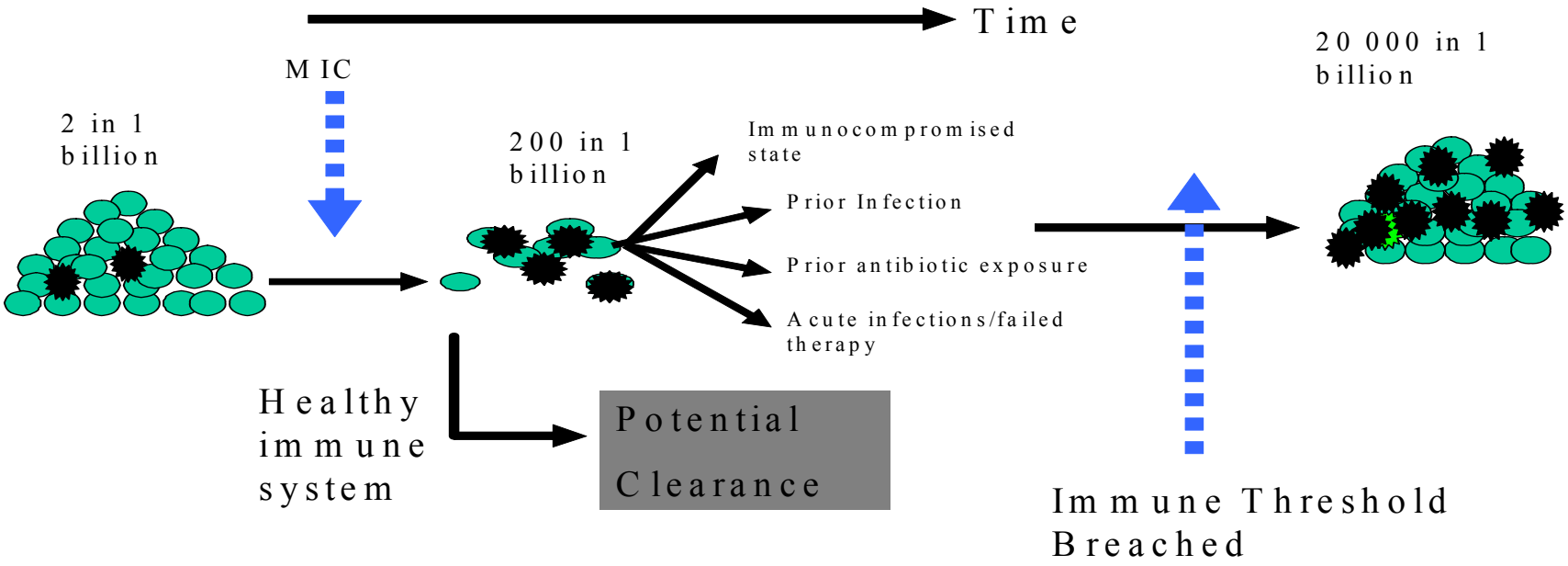
As the bacterial populations exceed the fluoroquinolone mutational frequency of 10^{-7} (10^{7} CFU/ml), mutant subpopulations (denoted by stars) arise. At concentrations >10^{9} CFU/ml, mutant populations which confer reduced susceptibilities are readily recovered. Infections containing >10^{9} cells will have many resistant mutants that are not inhibited the MIC. Current susceptibility tests utilize a dilution of the 0.5 McFarland standard which results in the application of 10^{5}-10^{6} CFU/ml during the testing process. This inoculm is too low to account for the presence of first-step resistant mutants present in large bacterial populations. The MPC reflects the antimicrobial concentration required to inhibit first-step resistant mutants when >10^{9} CFU/ml are tested (33, 144).

Experimentally, the MPC measurement can be measured as a characteristic two-stage decline in CFUs when large ($\geq 10^{10}$ CFU/ml) susceptible cultures are exposed to varying quinolone concentrations (83, 143). Increasing drug concentrations results in an initial sharp decline in colony recovery that occurs at concentrations at (or near) the MIC for wild-type susceptible populaton of cells. This first stage of colony decline occurs across a bacterial population of approximately 10^5-to-10^6 CFU/ml. At concentrations above the MIC of the wild-type strain, a plateau in colony recover occurs which corresponds to the outgrowth of mutant sub-populations. Mutant colonies require concentrations above the MIC of the wild-type strain in order to prevent their growth and a second sharp decline in colony recovery occurs when drug concentrations are reached which block the growth of all single-step mutants (41, 84, 149, 327). This later concentration defines the MPC. Thus, the MPC is a measure of the minimal antibiotic concentration required to prevent mutant growth recovered when large ($\geq 10^9$ CFU) numbers of cells are applied to antibiotic containing agar plates (83). In principle, the MPC represents a dosing threshold above which mutants should rarely arise; use of MPC would add consideration of the development of resistance to the traditional goal of clearing infection.

Conventional dosing strategies, based on the MIC as a measure of drug potency, allow concentrations to fluctuate within a range that enrich mutant fractions of heterogeneous pathogen populations. Thus, concentrations perceived to inhibit the majority of susceptible cells in cultures are the very concentrations that selectively enrich mutant subpopulations. If host defences fail to remove resistant mutants faster than they are

enriched, the mutant fraction will gradually increase (Figure 1.5). The concentration spectrum within which mutants are selectively enriched is termed the Mutant-Selection Window (MSW) and is defined by an upper and lower boundary based on the MPC and MIC, respectively (Figure 1.6) The MSW emerged from suggestions of Baquero *et al* (14) who indicated that a dangerous concentration range exists in which mutants are most frequently selected. The boundaries of the concentration range were later defined when the MPC measurements were determined for fluoroquinolones (41, 84, 142, 144, 149, 327, 367). When fluoroquinolone concentrations are below the MIC, neither susceptible cells nor first-step resistant mutants will be inhibited as the drug concentration is too low and the selective amplification of resistant subpopulations will not occur. When drug concentrations are in excess of the MPC, both susceptible and first-step resistant cells are inhibited and selective amplification of resistant cells does not occur. However, when drug concentrations fall within the MSW, the number of susceptible cells decline, thereby, creating conditions which result in the selective amplification of resistant populations. Selection of fluoroquinolone-resistant subpopulations will ultimately affect the entire fluoroquinolone class of antimicrobials because all agents share the small intramolecular targets. Thus, mutants selected by one compound will ultimalty affect the entire class. Continued use of antimicrobial agents against bacterial populations that have already been enriched for first-step mutants facilitates the selection of second- and third-step mutants (17, 85, 221), which confer even higher levels of resistance (41, 149, 370).

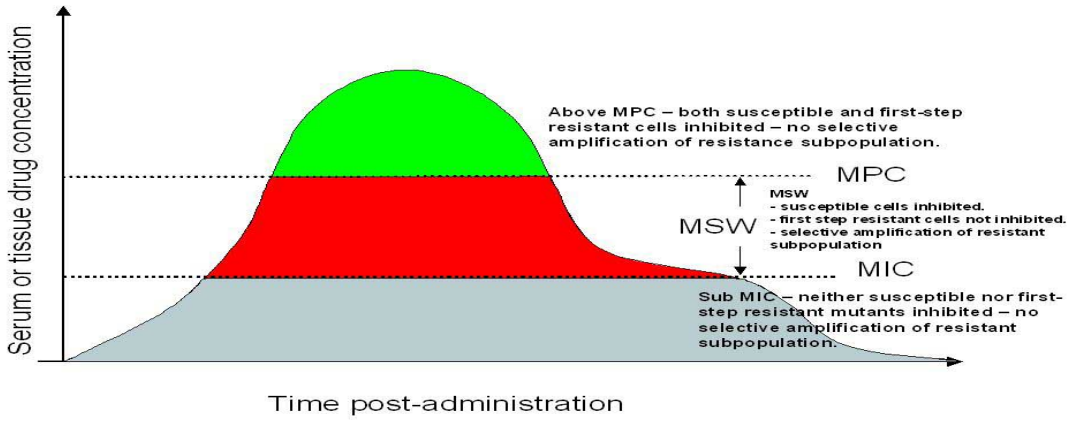
Figure 1.5 Selective Amplification of Resistant Mutants



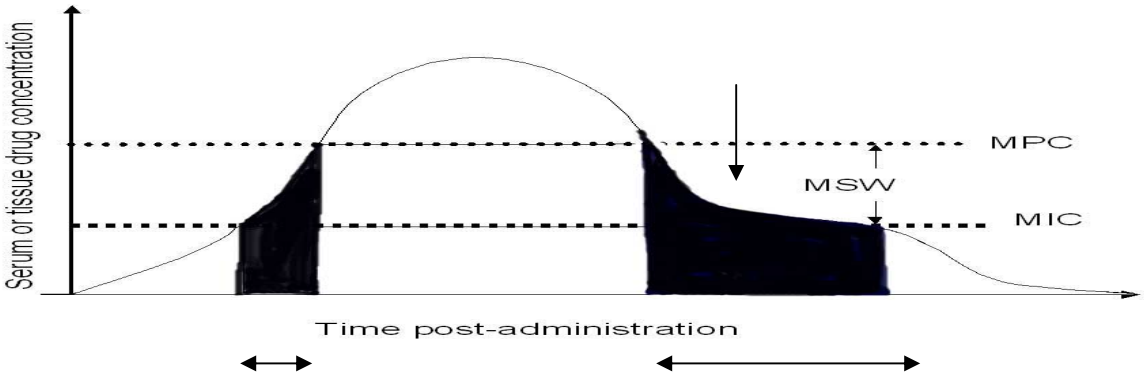
Bacterial populations may contain resistant cells which may become selectively enriched during antibiotic therapies if MPCs are not targeted during dosing. First-step resistant mutants (dark colored stars) are present in bacterial cultures containing between 10^7-to-10^9 CFU/ml. Increasing levels of resistance, concurrent/acute infections, and immunocompromised hosts place heavy reliance on the antimicrobial to control bacterial growth. Under these conditions resistant cells are selectively enriched during successive rounds of dosing. Eventually, the number of mutant cells may become high enough that mutant subpopulations become a dominant fraction of the bacterial population. If the infection has progressed to the point where the antimicrobial is incapable of directly controlling the bacterial growth then clinical failures and potential spread of resistant organism may occur.

Figure 1.6 The Mutant-Selection Window

A



B



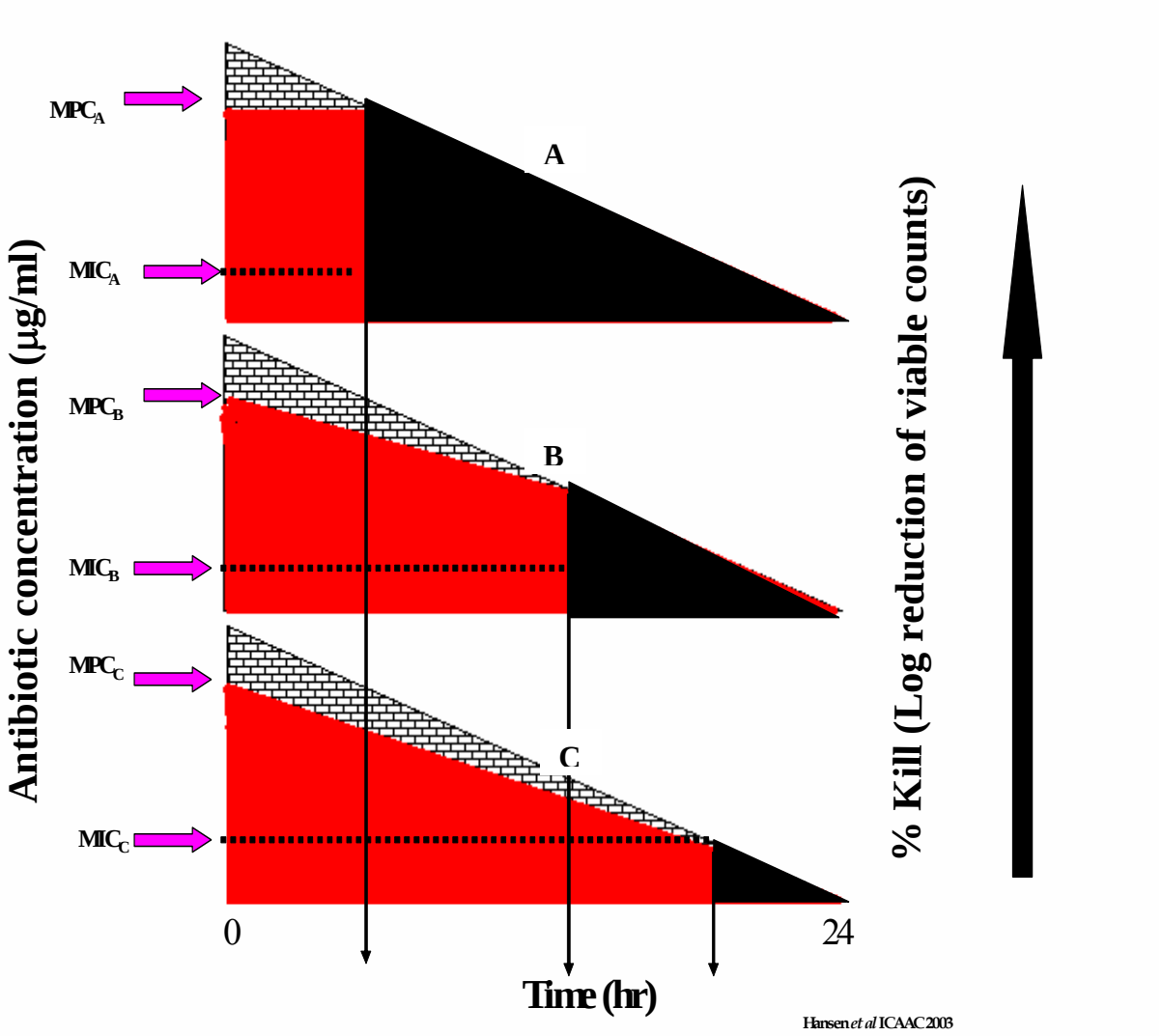
(A) The concentration spectrum extending from the MIC of the susceptible population to the MPC of the most-resistant, first-step resistant mutant present in culture represents the MSW. During dosing, concentrations which fluctuate within the window are expected to selectively enrich mutant populations. Concentrations above the MPC and below the MIC will not selectively enrich mutant populations because they offer no selective advantage. (B) A MSW will be created for every antimicrobial as concentrations rise and decline over a dosing period. Minimizing the length of time concentrations remain within the window will minimize the potential that resistant mutants will be selectively enriched.

Experimental evaluation of the MSW has recently been examined in an *in vitro* PD model (109). Firsov *et al* adjusted fluoroquinolone concentrations to mimic dosing regimes, which placed concentrations in one of three experimental categories: concentrations above the MSW, concentrations within the MSW and concentrations below the MSW. Dosing, which placed concentrations within the window for longer periods of time, resulted in the amplification of resistant mutants and decreases in susceptibility as assessed by elevated MICs (109). Despite suggestion to the contrary (328), the MPC and the MSW are not mutually exclusive, rather the MPC represents the upper boundary of the MSW. The relative size of the MSW for any fluoroquinolone-pathogen relationship is dictated by an upper and lower boundary, defined by the MIC of the susceptible population and the MPC for the most resistant, first-step resistant population. A large selection window suggests that mutant enrichment occurs over a wide drug concentration range and a larger fraction of the dosing interval. Compounds which exceed the MPC for the majority of the dosing interval and whose MIC and MPC closely correlate are expected to posses narrow selection windows and a reduced likelihood for selecting resistant cells (41, 85, 367). Ideally, dosing should be adjusted such that MPC concentrations are maintained throughout the dosing interval. However, it is important to understand that as antibiotic concentrations rise and decline upon administration and elimination of the antimicrobial, a MSWwill be created for any given "bug-drug" combination. Minimizing the time antibiotic concentrations fall within the MSW will be a key factor involved in minimizing the selection of resistant

cells. Determinations of the overall time each compound falls within the MSW may not be as straightforward as initially perceived because the size of the window will be influenced by the PK profile of the drug, the relationship of the MPC to the MIC and the rate of bacterial killing for a given antimicrobial agent. Thus, compounds with low MPCs may not necessarily be superior at preventing the selection of resistant mutants if MPC values do not fall within a clinically achievable range and for a long enough duration of the dosing period. As we learn more about the PK/PD properties of quinolones, such as implications of protein binding and site-specific drug accumulation, we will be able to refine the MWS concept.

The time that fluoroquinolone concentrations are required to exceed the MPC is a factor that is currently unknown. The answer to this question will ultimately lie in the rate at which fluoroquinolones are able to kill first-step mutants. For example, maintaining the MPC for half of a 12 hr dosing interval may be sufficient at preventing the selection of resistant mutants if all resistant mutants can be eliminated within the six-hr window (Figure 1.7). Bactericidal activity of different fluoroquinolones against selected first-step mutants will be an important discovery in the development of the MPC measurement and its potential clinical application. One recent study (66) has provided insight into how long the drug concentration needs to remain in excess of the MSW. Croisier *et al* (66) examined the *in vitro* pharmacodynamic efficacy of gatifloxacin against *S. pneumoniae* in an experimental model of pneumonia, specifically investigating the impact of low levels of fluoroquinolone resistance on the enrichment

Figure 1.7 The Relationship Between the MPC and Killing by Fluoroquinolones



The relationship between the MPC measurement and bacterial killing is illustrated. Bacterial cultures are represented by triangles. Susceptible and first-step mutant populations are represented by shaded and brick portions. Complete bacterial eradication of susceptible and mutant populations are shown as black filled portions. Cultures are treated with one of three hypothetical fluoroquinolones represented in A, B and C. In this example, all agents have comparable activity as depicted by identical MIC values (dashed lines). The MPC for each hypothetical fluoroquinolone are comparable. The rate, at which susceptible and first-step mutants are killed, differs with each fluoroquinolone (vertical arrows). The length of time the dose is required to exceed the MPC is dependent on the rate at which first-step resistant mutants are killed. The rate to bacterial eradication divided by the total time each agent is expected to exceed to the MPC should produce a value of ≤ 1 (T>MPC = $\leq$1). Values >1 could result in selective amplification of resistant sub-populations during therapy and may require higher, or more frequent dosing to control mutant populations.

of resistant mutants. They reported that when the time the drug concentration was within the MSW was >45%, the risk of selecting a mutant was 100%. Experimental evidence regarding the concept of MPC and killing will be discussed further in chapters 3 and 4.

The MPC is a new method for evaluating quinolone potency based on concentrations that restrict the selection of resistant sub-populations (32, 83, 85, 87, 144). I was interested in testing whether MPC measurements could be used to identify intrinsic differences between various fluoroquinolone antimicrobials which could be used to determine microbiological and clinical differences between the agents tested. In chapters 3-to-5, evidence is provided for the rationale approach for restricting the selection of resistant mutants based on the MPC principle using clinical isolates of *S. pneumonaiae, P. aeruginosa,* and Gram-negative enteric urinary pathogens. MPC measurements and the MSW hypothesis will be examined to identify differences in the anti-pneumococcal activities of newer generation fluoroquinolones based on their propensities for restricting the selection of resistant mutants. Many of these antimicrobial agents, such as levofloxacin, moxifloxacin and gatifloxacin have been deemed equivalent based on current perceptions of resistance and susceptibility testing. MPC results for clinical isolates of *P. aeruginosa* will be measured to see if the MPC and the MSW can provide insight into a number of unresolved issues relating to clinical differences among ciprofloxacin and levofloxacin in the treatment of *P. aeruginosa.*

Finally, MPC, MIC and MBC measurements for Gram-negative urinary tract pathogens will be described and discussed in relation to current measurements of susceptibility. MPC results for members of the *Enterobacteriace* will be further examined to see demonstrate if the ideas involved in the MPC measurement can be practically applied to fluoroquinolone therapies used in the treatment of UTIs.

2.0 MATERIALS AND METHODS

2.1 Standard Laboratory Methods

2.1.1 Isolate Collection and Identification

Most of the clinical isolates of *P. aeruginosa* used in this study were initially collected from 55 medical centers throughout Canada as part of a national surveillance study of *P. aeruginosa* (36-38) . The remaining strains were collected from February (2002) to September (2003) from the Clinical Microbiology Laboratory at Royal University Hospital, Saskatoon, SK. Oxidase-positive Gram-negative rods suspected to be *P. aeruginosa* were identified by accepted methods including API20 or Vitek (BioMerieux, St. Laurent, QC) systems. Three strains of *P. aeruginosa* (OCR1, MR27, MR2) overexpressing the MexAB-OprM (*nalB)* and MexEF-OprJ (*nfxC)* MDR efflux pumps, one strain possessing an altered outer porin profile (OprD), as well as the respective isogenic strain (PAO1), were kindly provided by Dr Xian-Zhi Li (Queen's University, Kingston, ON) and Dr. Satoshi Ohya (Sankyo, Co., Ltd., Tokyo, Japan) (232). A MexCD-OprN (*nfxB)* efflux mutant (K385) along with the isogenic strain, (K372) were obtained from the laboratory of Dr. Keith Poole (Queen's University, Kingston, ON). The antibiotic susceptibility patterns of efflux mutants of *P. aeruginosa* are reported in chapter 5 and elsewhere (232, 293).

Isolates of *S. pneumoniae* were collected from the Clinical Microbiology Laboratory, Royal University Hospital, Saskatoon, SK from 1998 to 2003 (41). No preselection criterion was used that would favour inclusion or exclusion of resistant

isolates and care was taken to avoid obtaining more than one isolate from a given patient. Identification of *S. pneumoniae* was preformed using traditional methods, including catalase testing, bile solubility and optochin sensitivity (250). A novel method called the Slidex Pneumo-Kit (BioMerieux), used in accordance with the manufacturer's instructions, was used to identify clinical isolates of *S. pneumoniae*.

Clinical strains of *C. freundii, E. cloacae, E. coli* and *K. pneumoniae* used in this study were part of an earlier study of Gram-negative urinary isolates (39) and were collected from medical centers throughout Canada. Identification of organisms was preformed in each participating hospital based on the reference method of the American Society for Microbiology and the NCCLS standards for identification of *Enterobacteriaceae* spp.

American Type Culture Collection (ATCC) strains for *P. aeruginosa* (ATCC 27853) and *S. pneumoniae* (ATCC 49609) were obtained from the American Type Cultures Collection (Rockville, MD).

2.1.2 Storage of the Bacterial Isolates

The isolates were obtained from Tryptic Soy Agar (TSA) slants (in house), TSA plates containing 5% sheep blood. Organisms identified as *S. pneumoniae* were streaked for isolated colonies on a TSA plate containing 5% sheep blood and incubated in 5% CO_2 at 35-to-37˚C for approximately 24 hr. One or two isolated colonies were selected from the TSA plate with a sterile wooden applicator stick and inoculated into 1.2 ml Corning cryovials containing 0.5 ml of skim milk. The vials were stored at -70˚C.

2.2 Susceptibility Testing

Antimicrobials used for *in vitro* experiments were obtained in powdered form and diluted in accordance with the manufacturers' recommendations. Sources of antimicrobials were as follows:

Moxifloxacin, ciprofloxacin – Bayer Pharmaceutical, West Haven CT;
Levofloxacin – The R. W. Johnson Pharmaceutical Research Institute, Raritan, NJ;
Gatifloxacin – Bristol Myers Squibb, Montreal, QC;
Grepafloxacin – Glaxo Smith Kline Pharmaceuticals, Collegeville, PA;
Trovafloxacin – Pfizer Canada, Kirkland, QC;
Garenoxacin – Bristol Myers Squibb, Montreal, QC;
Gemifloxacin – Glaxo Smith Kline Pharmaceuticals, Collegeville, PA;
Ceftazadime – Glaxo Smith Kline Pharmaceuticals, Collegeville, PA;
Gentamicin – Sigma-Aldrich Co., St. Louis, MO; and
Tobramycin - Sigma-Aldrich Co., St. Louis, MO.

2.2.1 Broth Microdilution

MIC values were determined by the broth microdilution test for *C. freundii, E.coli, E. cloacae, K. pneumoniae, P. aeruginosa,* and *S. pneumonaie* in accordance with the Natinal Committee for Clinical Laboratory Standards (NCCLS) guidelines. Ninety-six well flat bottom microtitre plates were filled with 100 µl of Mueller Hinton Broth (MHB) in each well of columns 2-to-12. Antimicrobial agent was serially diluted down the plate with column 1 containing the highest drug concentration and column 12 receiving no drug (growth control). Each isolate was standardized to a 0.5 McFarland suspension (~1.0 X 10^8 CFU/ml) using a colorimeter. The bacterial suspension was then diluted 1/100 with MHB (~1.0 X 10^5 to 10^6 CFU/ml). One

hundred µl of diluted cells were added to each well on the plate, resulting in a final volume of 200 µl. Purity of the bacterial suspension was confirmed by plating each sample onto a fresh TSA plate containing 5% sheep blood. The plates (both microtitre and blood agar) were incubated in ambient air at 35-to-37˚C for 16-to-20 hr. The growth control wells (column 12) were examined prior to MIC determination to ensure organism viability. The ATTC strain 27853 was used as a control to confirm accuracy of each MIC test. The MIC value was recorded as the lowest drug concentration at which there was no visible growth of the organism.

Broth microdilution for *S. pneumoniae* isolates was done similar to that described for Gram-negative organisms, except Todd Hewitt Broth (THB) was used as was the *S. pneumoniae* ATCC control strain, 49619. Microtitre trays were incubated at 35-to-37°C in 5% CO_2 for 16-to-20 hr. Susceptible, intermediate, or resistant phenotypes were recorded based on current NCCLS breakpoints.

2.2.2 Broth Microdilution using CCCP

Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) has been reported to increase the intracellular accumulation of antibiotics, including quinolones, in bacterial cells (172, 230, 287, 300, 365). Early investigators (64, 192) theorized that CCCP blocks an energy-dependent efflux system for quinolones with a consequent increase in quinolone concentration. The CCCP effect has also been interpreted to be the result of a perturbation of the outer and the cytoplasmic membranes resulting in the influx of quinolones into bacteria (77, 287). This was proposed to result from a collapse in the pH gradient of the cytoplasmic membrane by CCCP (265). A 2-to-4 fold decrease in

the MIC in the presence of CCCP has been interpreted to indicate that an active efflux mechanism is inhibited (209). Stock solutions (50 mM) of CCCP were dissolved in methanol and stored at −20°C. A 200 μM solution of CCCP was added to the wells of the microtitre panels used in the microbroth dilution method for susceptibility testing. Panels containing organism, drug, and CCCP were incubated at 35-to-37°C in ambient air for 16-to-20 hr. A 2-to-4 fold decrease in the MIC upon the addition CCCP was taken as a marker for a possible efflux mechanism.

2.2.3 MBC Testing

The MBC defines the lowest antimicrobial concentration that kills 99.9% of a standardized 10^5 to 10^6 CFU/ml bacterial culture. The MBC is performed using the microbroth method for susceptibility testing. At an 18-to-24 hr endpoint, a 50 μl aliquot of each well (containing a specified antimicrobial concentration) for each isolate tested was applied to a TSA plate containing 5% sheep blood and incubated at 35-37°C in ambient air for 18-24 hr. Resulting growth (or lack of growth) was examined after 18-to-24 hr and the lowest concentration that inhibits 99.9% of the original culture was calculated and taken as the MBC.

2.2.4 E-Test

E-tests were used to determine whether MPC testing (see section 2.2) could be applied to conventional susceptibility tests. Three TSA plates containing 5% sheep blood were inoculated with *P. aeruginosa* to produce confluent lawns of bacterial growth and incubated at 35-to-37°C in ambient air for 18-to-24 hr. After 24 hr, the contents of the plates were inoculated into 150 ml of fresh MHB, followed by

incubation for 16-to-20 hr at at 35-to-37^{o}C in ambient air. Cells were collected via centrifugation at 6000 rpm for 20 mins at 4°C. The contents of the culture were suspended in 4 ml of fresh MHB and viable counts were performed to determine the number of cells per ml. A sterile cotton swab was inoculated into the bacterial suspension and applied to TSA plates containing 5% sheep red blood cells. Three E-test strips for ciprofloxacin, containing a gradient of antimicrobial concentrations, were applied in an overlapping fashion across the plate. The corresponding concentration that inhibited bacterial growth was taken as the MIC.

2.2.5 Vitek Results

A semi-automated clinical laboratory organism identification/susceptibility system known as Vitek (BioMeriux) is often utilized in clinical laboratories for identification and susceptibility testing of bacteria. Identification cards contain various carbohydrate and biochemical substrates and when inoculated with organisms, positive and negative reactions are interpreted by the instrument (following incubation) and identification is reached by comparison of a computerized database of profiles. For susceptibility cards, organism is inoculated to various drug concentrations and following incubation, growth or inhibition in reference to the drug concentrations is examined and compared to standardized curves whereby susceptibility or resistance is determined. Cultures of *P. aeruginosa* were grown in MHB to cellular concentrations of $\geq 10^{10}$ CFU/ml. Cells were collected via centrifugation at 6000 rpm for 20 min at 4°C and resuspended in 4 ml of fresh MHB. One hundred µl of culture were transferred to the wells of the Vitek card (GNS-618 V4423) which was placed into the

Vitek system. A printout of the respective susceptibilities against 16 different antimicrobials was recorded after an elapsed time of 7 hr. The same procedure was repeated using a 0.5 McFarland standard.

2.2.6 Agar Dilution

Agar dilution was performed in accordance with NCCLS guidelines for susceptibility testing (258). Each isolate was standardized to a 0.5 McFarland suspension (~1.0 X 10^8 CFU/ml) using a Colorimeter. The bacterial suspension was then diluted 1/100 with MHB (~1.0 X 10^5 to 10^6 CFU/ml). One hundred µl of diluted cells were added to a series of TSA plate containing 5% sheep blood seeded with two fold increments of antimicrobial agent. Purity of the bacterial suspension was confirmed by plating each sample onto a fresh TSA plate containing 5% sheep blood. Plates were incubated in ambient air at 35-to-37˚C for 16-to-20 hr. The MIC was recorded as the lowest antimicrobial concentration that inhibited growth**.**

2.3 Mutant-Prevention Concentration (MPC)

The MPC measurements for clinical isolates of *C. freundi, E. cloacae, E. coli, K. pneumoniae, P. aeruginosa* and *S. pneumoniae* (41, 144, 145, 149) were developed in the laboratory of Dr. J. Blondeau, Royal Univerisity Hospital, Saskatoon, SK.

2.3.1 Inoculum Preparation and MPC Testing Procedure

Strains of *C. freundii, E.coli, E. cloacae,* K. *pneumoniae,* and *P. aeruginosa,* were subcultured from thawed skim milk onto TSA plates containing 5% sheep blood using a sterile wooden applicator stick and incubated in ambient air at 35-to-37˚C for approximately 24 hr. After overnight incubation, a sterile swab was used to create

heavy lawns of bacterial growth on three TSA plates (containing 5% sheep blood) for each isolate. Plates were incubated in ambient air at 35-to-37˚C for approximately 24 hr. An initial absorbance reading of $\geq$ 1.00 at a wavelength of 600 nm was used to estimate a cellular density $>10^{10}$ cells. Cells were collected via centrifugation in an Avanti J-E centrifuge (Beckman Coulter, Fullerton, CA, USA) at 5000 x *g* for 30 min at 4°C and resuspended in a total of 4 ml of cold, fresh MHB broth. Viable counts were performed on the high-density bacterial cultures to confirm the presence of $\geq 10^{10}$ CFU/ml. Three 100 µl aliquots of $>10^{10}$ CFU/ml cultures were applied to TSA plates containing 5% sheep red blood cells. For each experiment, agar dilution plates were prepared by incorporating fluoroquinolones at seven concentrations into the TSA agar-sheep red blood cell plates (plates were stored at 4°C and used within 7 days of preparation). Each experiment included the fully susceptible control strain *S. pneumoniae* ATCC 27853 grown to a standardized inoculum of 1.1 $x10^{10}$ CFU/0.1 ml. Inoculated plates were incubated for 24 hr at 35-to-37°C in ambient air (or 5% CO_2 for *S. pneumoniae*) and then screened for growth. All plates were reincubated for an additional 24 hr and re-examined. The MPC was recorded as the lowest antibiotic concentration that allowed no growth.

For *S. pneumoniae*, starter cultures were spread on blood agar plates (six plates per isolate) (PML, Richmond, ON) and incubated overnight (18-to-24 hr) at 35-to-37°C in 5% CO_2. Bacterial cells were then transferred from the plates to 500 ml of THB (Difco Laboratories, Detroit, MI) followed by overnight incubation at 35-to-37°C in 5% CO_2. After incubation, cultures were estimated to have concentrations of 3×10^8

CFU/ml by absorbency (>0.4 at 600 nm). Cultures were concentrated by centrifugation at 5,000 × *g* for 30 min at 4°C and re-suspended in 3 ml of THB. Aliquots of 200 µl, containing $\geq 10^9$ CFU, were applied to TSA plates containing 5% sheep red blood cells. For each experiment, agar dilution plates were prepared by incorporating fluoroquinolones at seven concentrations into the TSA plates containing 5% sheep red blood cells and plates were stored at 4°C and used within 7 days of preparation. Each experiment included the fully susceptible control strain *S. pneumoniae* ATCC 49619 grown to a standardized inoculum of $5x10^9$. Inoculated plates were incubated for 24 h at 35-to- 37°C in 5% CO_2 and then screened for growth. All plates were re-incubated for an additional 24 hr and re-examined. The MPC was recorded as the lowest antibiotic concentration that allowed no growth.

Gram-negative organisms readily achieve high bacterial titres, in excess of 10^{10} CFU/ml, which allows MPC measurements to be preformed using small (between 100-to-300 µl) inoculums and relatively few MPC plates. For fastidious organisms, such as *S. pneumoniae* and organisms which do not easily achieve high bacterial concentrations, higher volumes of organism (300-to-400 µl) or additional plates for each concentration tested were required to ensure that 10^9-to-10^{10} cells were tested. Mutant selection curves should be performed to ensure that a high enough number of cells are tested in order to capture representative first-step mutants which can be visualized by a characteristic two-stage decline in CFU that occurs in cultures containing in excess of 10^7-to-10^8 cells.

In some instances, a lack of visible growth could not be accurately ascertained after 48 hr and a faint haze caused by the inoculum may obscure growth. A sterile cotton swab was used to collect potential bacterial growth and was inoculated into a test tube containing 200 µl of fresh THB. The tube was briefly vortexed and the entire concents of the tube were applied to TSA plates containing 5% sheep red blood cells and the fluoroquinolone concentration in question. Plates were incubated under appropriate conditions for an additional 18-to-24 hr period to confirm the absence of growth. For most strains tested, a lack of visual growth after 48 hr correlates with a confirmed lack of growth after the additional 24 hr incubation. The concentrations tested in the MPC method include one doubling dilution below the MIC of the isolate and a minimum of five doubling dilutions above the MIC. Upon repeat testing, the range of drug concentrations varied depending on the behavior of the organism (if the organism did not grow on any antimicrobial plates, the MIC was repeated and the MPC range was lowered; if the organism grew at the highest drug concentration plate, the MPC range was extended and the experiment was repeated at a higher drug concentration range). For instances in which absolute growth could not be accurately determined by 48 hr, a swab of the plate was transferred to a 1-to-2 ml test tube containing fresh media and the contents of the tube were applied to fresh agar seeded with the corresponding drug concentration from which the swab was taken. The plate was incubated for an additional 18-to-24 hr (72 hr total) and the lowest concentration producing a lack of visible growth was recorded as the MPC.

2.3.2 Mutant Selection Curves

Overnight cultures of bacteria (typically 3-to-6 plates/isolate), grown under appropriate conditions for the organism, were inoculated in liquid media and incubated for an 18-to-24 hr period. After incubation, cells were collected via centrifugation at 5,000 × *g* and 4°C for 30 min and then re-suspended in 6 ml (or calculated volume required) of fresh MHB/culture. The contents of the two high-density cultures were pooled and a viable count of the culture was performed to ensure the presence of $\geq 10^{10}$ CFU/ml. Dilution sets consisting of 1/10 to $1/10^{8}$ were created and 100 µl aliquots of various dilutions and pure culture were applied in triplicate to TSA plates seeded with fluoroquinolone concentrations spanning small concentration intervals (i.e., <1 doubling dilution). The decline in colony recovery was measured at each concentration and used to create the overall mutant selection curve(s). For fastidious organisms such as *S. pneumoniae*, a total of 1 ml was sampled in 200 µl aliquots, containing approximately 10^{10} cells) over 5 different plates to ensure that 10^{10} cells were tested. The concentration intervals tested should be designed to illustrate a distinct plateau region, followed by a second decline in colony recovery. Thus, concentrations tested varied from organism to organism and strain-to-strain depending on the MPC of the strain tested.

2.3.3 Viable Counts

Viable counts were performed on selected organisms for each round of MPC testing. Dilutions of 10^{-7}, 10^{-8} and 10^{-9} were made from the bacterial suspension and 100 µl of each dilution were plated onto triplicate TSA plates containing 5% sheep

blood. The plates were incubated for 24 hr at 35-to-37°C in ambient air. Colony counts were performed on each plate. Calculations were then performed to determine CFU/ml.

2.4 Characterization of First-step Mutants

2.4.1 DNA Isolation, Amplification and Nucleotide Sequence Determination for *S. pneumoniae*

Selected isolates of *S. pneumoniae* were grown on brain heart infusion (BHI) agar (Difco) containing 10% defibrinated sheep blood (Hemostat Laboratories, Dixon, CA.) following high-density inoculation. Incubation was overnight at 35-to-37°C in 5% CO_2. Bacteria grown as confluent lawns were recovered from agar plates by washing with 2 ml of THB per plate. Cells were concentrated by centrifugation (as previously described), washed once with lysis buffer (50 mM Tris-HCl [pH 8.0] and 5 mM EDTA) and resuspended in 400 µl of lysis buffer per plate. Then, 50 µl of 10% sodium dodecyl sulfate and 20 µl of proteinase K (10 mg/ml) were added and the mixture was incubated, first at 55°C for 30 min and then at 37°C for 1.5 hr. Cell lysates were extracted with phenol. An equal volume of TE-saturated phenol (10 mM Tris-HCl [pH 8.0] and 1 mM EDTA) was added to 100 to 200 µl of DNA sample contained in a 1.5 ml microcentrifuge tube and vortexed for 15-to-30 sec. The sample was centrifuged at 10,000 rpm for for 5 min at room temperature to separate the phases. The upper aqueous layer was aspirated, and placed in a clean tube, and was extracted a second time. DNA was precipitated with ethanol and recovered by centrifugation. DNA was then dissolved in TE buffer and treated with a final concentration of 100 µg of RNase A per ml for 1 hr at 37°C. DNA was reprecipitated with 2 volumes of ethanol and

dissolved in TE buffer.

The nucleotide sequences of the quinolone-resistance-determining regions of *parC* and *gyrA* were determined with an automated DNA sequencer (Applied Biosystems, Foster City, CA) using primer SP-*parC* sequence (5' TCA GCG CCG TAT TCT TTA TTC TAT G 3') and primer SP-*gyrA* sequence (5' TCG AGA TGG CTT AAA ACC TGT TCA C 3') after PCR amplification of DNA fragments using primers SP-$parC_{fwd}$ (5' GTC TAA CAT TCA AAA CAT GTC CCT G 3'), SP-$parC_{rev}$ (5' TCT TTC TCC GTA TCG TCA AAG TTC 3') for *parC* and SP-$gyrA_{fwd}$ (5' TGT CAA TCT GAC AAA GGA GAT GAA G 3') and SP-$gyrA_{rev}$ (5' CCA GTT GCT CCA TTA ACC AAA AG 3') for *gyrA*.

PCR reactions were performed in 100 μl volumes containing 0.1 μM of each nucleotide primer, 0.2 μM each 2'-deoxynucleoside 5'-triphosphate (Pharmacia, Montreal, QC), 10 μl of reaction buffer (Sigma-Aldrich), 4 μl of template DNA sample and 1U of platinum Pfx polymerase (Gibco BRL, St. Louis, MO). All reactions were performed in a DNA thermocycler (MJ research, Waltham, MA) and cycled 25 times following a 6-min denaturation step at 94°C. Each cycle consisted of a 30 sec denaturation step at 94°C, a 30 sec annealing step at 55°C and 2 min at 72°C for primer extension. PCR products were purified using a QIAquick PCR purification kit (Qiagen Inc., Mississauga, ON) according to the manufacturers' recommendations. DNA sequencing was performed using an automated gene sequencer (Natural Research Council, Saskatoon, SK).

2.4.2 DNA Isolation, Amplification and Nucleotide Sequence Determination for Recovered Mutants of *P. aeruginosa*

Selected mutants of *P. aeruginosa* were recovered from MPC plates seeded with ciprofloxacin and levofloxacin. Two colonies were selected and suspended in 100 µl of insta-gene matrix (Sigma-Aldrich). Cells were centrifuged for 15 sec and boiled for 10 min. Following a second centrifugation step, 4 µl of the supernatant was used for PCR reactions.

Two primers GYRA1 (5' CCAAAGAAATTCTCCGGTCA 3') and GYRA2 (5' GGTTGGGAATCTTGGTCGGCA 3') were constructed based on the nucleotide sequence of *P. aeruginosa* gyrA subunit reported by Kureishi *et al* (196). Primers for DNA gyrA were designed to amplify a 484 base pair region spanning nucleotides 256-740. This region includes the QRDR encompassing codons 67-to-106. One 22-mer primer and one 21-mer primer (PARC1 5' ATGAGCGCCTCCCTCGATCTGA 3'and PARC2 5' GCCGRCGAAGRRCGGRACCCA 3') were constructed based on the sequence of the *P. aeruginosa* Topoisomerase IV gene reported by Akaska *et al* (2). The PCR reaction amplified a 424 base pair region including the QRDR associated with Topoisomerase IV.

PCR reactions were perfomed as previously described for *S. pneumoniae.* PCR reactions cycled 36-to-41 times following a 6-minute denaturation step at 95^{o}C. Each cycle consisted of a 35 second denaturation step at 94^{o}C, a 40 second annealing step at 57^{o} C and a 35 second polymerization at 72^{o}C. PCR products were purified and sequenced as previously described for *S. pneumoniae*.

2.4.3 Primer Preparation and Storage

Each primer set was made at a stock 100 mmolar concentration using TE buffer. Primers were stored in aliquots at –20^{o}C.

2.4.4 Analysis of PCR Products

PCR products were analyzed on 1% agarose gels prepared as described in Appendix A. Six µl of tracking dye were added to each PCR reaction tube. For the marker, 2 µl of a 123 bp ladder (Sigma, St. Louis, MO.) were added to 12 µl of sterile distilled water and 4 µl of tracking dye. Each tube was gently mixed. A sample volume of 20 µl was loaded into designated lanes and a current of 117 volts was applied to the gel for approximately 20-to-30 min. Gels were analyzed under ultraviolet light.

2.5 Pulsed-Field Gel Electrophoresis (PFGE) for Clinical Isolates of *P. aeruginosa*

2.5.1 DNA Extraction

P. aeruginosa isolates were thawed and plated out on TSA supplemented with 5% sheep red blood cells. Plates were incubated at 35-to-37^{o}C for 18-to-24 hr in ambient air.

2.5.2 Cell Disruption

An overnight culture was used to create a bacterial suspension in TE buffer. An inoculum equal to a 5.0 McFarland standard (~15 x10^{8} CFU/ml) was created. A 1.5 ml aliquot of the bacterial suspension was then mixed with an equal volume of a 1.2% low melting point agrose solution. The mixture was vortexed for 20 sec and aspirated into a 3cc monoject Luer lock syringe with a 1.5 gauge needle (Fisher Scientific, Napean,

ON). The syringes were incubated at 4°C for 30 min. The solid agarose cylinder was removed from the syringe and placed in a sterile plastic petri dish. Using a sterile scalpel blade, the cylinder was cut into 10-to-15 (approximately 3 mm in thickness) agarose plugs. The plugs were added to sterile test tubes containing 4 ml of proteinase K lysis buffer and incubated at 50°C with constant agitation.

2.5.3 Proteinase K Treatment

Most standard protocols for DNA extractions from Gram-negative bacteria for PFGE require 4-to-6 hr Proteinase K incubation. However, for *P. aeruginosa,* an incubation of this length is often insufficient to obtain the required cell lysis. Increasing the amount of time the Proteinase K was able to react from 6 hr to overnight (16-to-18 hr) increased lysis efficiency (Hansen and Blondeau-unpublished observations). The Proteinase K lysis buffer was removed and replaced with 4 ml of lysis buffer and incubated for a further 24 hr at 50°C

2.5.4 Inactivation of Proteinase K

Lysis buffer was removed and replaced with 4 ml of phenylmethyl-sulfonyl fluoride (PMSF) rinse solution. This rinse step was performed at least twice with continuous agitation at room temperature for 1 hr each time. The PMSF rinse solution was replaced with 4 ml of TE buffer and agitated for 30 min at room temperature. This rinse step was repeated three times.

2.5.5 Storage of Plugs

The TE buffer rinse solution was removed from the plugs and the plugs were stored in 3 ml of fresh TE buffer in tissue culture plates (Fisher Scientific Nepean, ON)

at 4°C. The level of TE buffer was monitored and replenished frequently to prevent the plugs from drying.

2.5.6 Restriction Endonuclease Digestion

Plugs were removed from storage plates using a sterilized spatula and placed in a sterile plastic petri dish. Individual plugs were cut into quarters, with one quarter being transferred to a sterile 1.5 ml microcentrifuge tube. A 1 ml mixture of the manufacture's 1x restriction endonuclease buffer, bovine serum albumin and 14 µl (between 30 and 270 units) of the enzyme SpeI was added.

2.5.7 Preparation of the Gels

A 1% pulsed-field certified agarose gel was prepared in a 250 ml flask. The molten agarose was cooled to 50-to-55°C and the gel was cast in a BioRad Laboratories pulsed-field gel casting apparatus. The agarose was allowed to set for 30 min after which the molten plugs were transferred to the wells in the gel. The last well was reserved for the λ ladder molecular weight marker. The gel was submerged in PFGE running buffer (Appendix A) in the electrophoresis cell of a BioRad CHEF DR III system. The gel was subjected to pulsed orthoganol fields ramping from 2-to-15 sec, opposing each other by 120° at a field strength of 6 V/cm. The buffer was held at a constant temperature of 14°C by a cooling unit. The electrophoresis was carried out over 18 hr.

2.5.8 Staining Procedure

A 1 mg/ml solution of ethidium bromide solution was placed in a plastic tupperware container containing distilled water, the gel was then removed from the

electrophoresis cell and placed in solution. The gel was protected from direct light and agitated for 30 min. The stain solution was decanted and the gel destained for 30 min in sterile distilled water with agitation. The gel was analyzed on the Gel Doc 1000 Illuminator (BioRad Laboratories, Mississauga, ON). A difference of three or more bands is typically used to distinguish between different clones (137, 138) and was used as the criteria for distinguishing between different strains.

2.6 Killing of *S. pneumoniae* by Fluoroquinolones Assessed with Conventional Kill Curves

Powdered forms of each fluoroquinolone were dissolved according to the manufacturer's instructions. Stock solutions were prepared from fresh preparations or samples stored at –70°C. Clinical isolates of *S. pneumoniae* used in kill curve experiments were grown overnight on blood agar plates. The following day, an inoculum was transferred to THB and incubated for 2 hr at 35-to-37°C in 5% CO_2. After incubation, spectrophotometer readings of $\geq$ 1.5 (at 600 nm) were used to verify cell densities of $\geq 10^9$ cells/ml as previously determined. To achieve cell densities ranging from 10^6 to 10^9 cells/ml, further dilutions in THB were taken. The antimicrobial agent was then added. Drug concentrations used for kill experiments were based on the MIC, MPC and C_{max} drug concentrations. The measurement of kill (log reduction in viable cells) was recorded at 0, 0.16, 0.5, 1, 2, 3, 4, and 24 hr after the addition of the drug. Three 100 µl aliquots were taken at each time interval and cultured on drug-free agar plates that were subsequently incubated overnight at 35-to-37°C in 5% CO_2. The three results obtained at each time interval were averaged and the $\log_{10}$ reductions and percent killing of viable cells were calculated and recorded.

Significant bactericidal activity was recorded as ≥ -3 log reduction in viable cells, as previously established in the literature (34, 245).

2.7 Killing of *P. aeruginosa* by Ciprofloxacin and Levofloxacin Assessed with MPC-based Kill Curve Experiments

Kill curve experiments designed to compare the effects of MPC and MIC based-killing were performed with clinical isolates of *P. aeruginosa*. MIC and MPC measurements were preformed as previously described (Section 2.3). Preparation of high-density *P. aeruginosa* cultures were prepared as outlined in MPC testing (see Section 2.2). Viable counts were performed on the high-density culture to determine the number of cells present per ml of culture. Cultures were re-suspended in 4 ml of fresh cold MHB and divided equally into four flasks containing 500 ml each of fresh MHB broth. For ciprofloxacin and levofloxacin, two separate flaskes containing the contents of the initial 10^{10} CFU/ml were used for each agent and tested for killing at either the MIC of MPC, respectively. This procedure distributed the contents of the $>10^{10}$ CFU/ml culture into 4 cultures, each containing approximately 10^7 cells. Two flasks (culture 1 and culture 2) were dedicated for use with either ciprofloxacin or levofloxacin at the measured MIC or MPC drug concentration. Three aliquots containing 300 μl were sampled from each flask at 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 and 24 hr and applied to TSA supplemented with 5% sheep red blood cells and incubated for 24 hr while shaking at 35^o C in ambient air. A dilution series was created for each flask at each time interval sampled to ensure that individual colonies could be counted. An additional 300 μl sample was taken from each flask and applied

to TSA supplemented with 5% sheep red blood cells and seeded with ciprofloxacin or levofloxacin at concentrations one dilution below the MPC value of the strain tested.

Bacterial growth at each time interval for each flask was recorded and the log growth was subtracted from the initial cell density of starting culture before the addition of drug. Mutant growth, as assessed by growth on plates seeded with fluoroquinolone concentrations 2-fold below the measured MPC, was recorded. After 24 hr, cells were collected from each culture via centrifugation at 5000 x g for 30 min at 4°C and re-suspended in a volume of 5 ml of fresh MHB. The contents of the entire cultures were sampled in 0.3 ml aliquots on TSA plates supplemented with 5% sheep red blood cells and seeded with the fluoroquinolone concentration that corresponded to one dilution below the MPC value of the strain. This final process was used to determine the viability of mutant cultures.

2.8 Ciprofloxacin and Levofloxacin Efficacy in a Rat Abscess Model of *P. aeruginosa* Infection

Individual strains of *P. aeruginosa* were plated on three TSA plates supplemented with 5% sheep red blood cells and grown overnight (18-to-24 hr) at 35-to-37^{o}C in ambient air. After 24 hr, cells were collected via centrifugation at 5000 x *g* at 4^{o}C for 30 min and re-suspended in a total of 4 ml of cold, fresh MHB broth. Viable counts were preformed on the high-density bacterial cultures to confirm the presence of > 10^{10} CFU/ml. Three sterile sections of open-ended dialysis tubing (~1.5 cm in length) were inoculated with 50-125 µl of concentrated *P. aeruginosa* culture using a sterile pipette. Each implant contained between 5 x10^{9} to 4.3 x10^{10} CFU/ml as determined by viable counts.

2.8.1 Animal Model of Infection

Male Sprague Dawley white rats (Charles River, Wilmington, MA) weighing between 300 and 350 g were used. Sixteen rats were used per experiment set, which included 4 control animals (2 animals did not receive drug, 2 animals were implanted with saline- filled implants), 6 ciprofloxacin-treated animals and 6 levofloxacin-treated animals. Rats were weighed and anaesthetized with an intra-peritoneal (I.P.) injection using a 1cc syringe with a 25 GA needle containing a 90 mg/kg:5 mg/kg ketamine:zylazine dose (Warner-Lambert and Co., Bellevelle, ON) (Bayer, Toronto, ON). Animals were placed on a warm protected surface for approximately 5-10 min. A drop of eyelube (Sabex, Boucherville, QC) was administered to each eye. A 5 cm x 5 cm long section of the dorsal flank of each rat was shaved, scrubbed with hibitane (Fisher scientific, Nepean, ON) and washed with 95% alcohol. Under anaesthetic, an elastic band was tied around the base of the tail and a rat tail vein catheter (22 GA x 2.5 cm I.V. catheter, Becton Dickinson Infusion Therapy System Inc., Sandy, UT,) was inserted. A protective gauze wrap was inserted over the catheter to prevent the rat from removing it. In each rat, three 1.5 cm long incisions were made in a triangular fashion (beneath each scapula and on the midline of the lower back) using a size 10 sterile disposable scalpel (Fisher Scientific, Nepean, ON). The skin was separated from the fascia by blunt dissection using sterilized forceps (Fisher scientific, Nepean, ON). The implants were positioned in a triangular pattern behind the shoulders. The overlying incision was sealed with a combination of individual 00 Vicryl silk sutures (Ethicon, Sommerville, NJ) and/or Vetbond tissue adhesive (3M Animal Care

Products, St. Paul, MN). A 0.05mg/kg injection of buprenorphine (Reckitt and Colman Pharmaceuticals Inc. Richmond, VG) was given by (I.P.) injection for pain control. Animals recovered on a clean, soft surface under a heat lamp for 15-to-30 min.

2.8.2 Administration of Fluoroquinolones

Antimicrobials were administered I.V. in 1 cc syringes with a 25 GA needle via the tail vein catheter as 400 mg ciprofloxacin (q24h and qh8) and 750 mg levofloxacin (q24h, q8h) doses. Doses were started 3-to-4 hr after the implantation of implants.

2.8.3 Recovery of Blood Samples

Blood samples were collected in 10-to-15 μl amounts, taken every 18-to-24 hr from a tail vien catheter and were followed by a 100-to-150 μl infusion of sterile saline solution to replenish fluid volume and flush residual antimicrobial from the site.

2.8.4 Recovery of Infected Tissue and Blood Samples

In an attempt to ensure that the majority of an animal's blood volume was recovered, a 20 cc syringe with a 20 GA needle filled with saline was introduced into the inferior section of the heart, inserted into the aorta and clamped with a 15.9 cm hemostat (Fisher Scientific, Neapon ON). The right ventricle was cut and sterile saline was infused throughout the circulatory system and collected in a tube attached beneath the left ventricle. Blood samples were injected into pediatric Bactec resin bottles (Becton Dickson, Sparks, MD) (which coagulates any remaining antibiotic) using a 20 GA syringe and incubated for 12t-to-24 hr at 35-to-37^{o}C in ambient air. Inoculated bottles were incubated in the Bactec 9000 blood culture system (Becton Dickson, Sparks, MD) and incubated with agitation for 5 days. Bottles flagging positive were

removed from the instrument and an aliquot of the contents were removed with a sterile needle and inoculated to agar plates and incubated for 18-to-24 hr at 35-to-37^{o}C in ambient air. The next day, culture plates were inspected for growth and recovered organisms were identified by the Vitek system. Culture-positive blood samples were streaked onto MaConkey and blood agar plates and incubated for 18-to-24 hr at 35-to-37^{o}C in ambient air. Bacterial growth was identified as *P. aeruginosa* on the based on colonial morphology, Gram stain, and a positive oxidase test. Infected tissues were recovered and homogenized with the use of a high-speed homogenizer (Labcor, Concord, ON). The resulting suspension was streaked for growth and any growth was identified as *P. aeruginosa* by Gram stain and a positive oxidase test. Recovered organisms were stocked, and frozen at –70^{o} C in sterile cryovials as previously described.

3.0 MUTANT-PREVENTION CONCENTRATIONS OF FLUOROQUINOLONES FOR CLINICAL ISOLATES OF *S. PNEUMONIAE*

3.1 Abstract

Antibiotic resistance among human pathogens now occurs in almost every bacterial species for which antibiotic therapies exist. In the case of *S. pneumoniae*, resistance to penicillin and macrolides (i.e., azithromycin, clarithromycin, erythromycin) has become so widespread that clinicians have started to use the fluoroquinolones for the treatment of community-acquired respiratory tract infections. Fluoroquinolones with increased anti-pneumococal activities are becoming available, but new treatment strategies must accompany use of these agents in order to halt the selection of resistant mutants. The MPC represents a concentration threshold above which the selective proliferation of resistant mutants is expected to rarely occur. MPCs were defined and measured for six fluoroquinolones with clinical isolates of *S. pneumoniae*. Based on their potential for restricting the selection of resistant mutants, the six fluoroquinolones, in descending order of potency, were found to be: gemifloxacin > moxifloxacin > trovafloxacin > gatifloxacin > grepafloxacin > levofloxacin. For several compounds, clinical isolates that lacked a known resistance mutation had an MPC value that was close to or below the serum levels that could be attained using current dosing strategies. These data identify moxifloxacin, gatifloxacin and gemifloxacin as good candidates for determining whether MPC can be used as a guide for choosing and eventually administering fluoroquinolones in a way that would significantly reduce the development of resistance. High MPC results for levofloxacin

suggest that more than once daily dosing may be required in order to ensure that drug concentrations remain in excess of the MPC for a sufficient amount of the 24 hr dosing period. Killing experiments based on the MPC and performed using conventional kill curve methodology require further examination but suggest that killing based on the MPC may be essential for rapid bacterial eradication and shorter durations of therapy.

3.2 Introduction

S. pneumoniae is the most common bacterial pathogen associated with (lower) respiratory tract infections and accounts for approximately 50% of all cases of community-acquired pneumonia, 35% of cases of acute sinusitis and acute otitis media and 20% of acute exacerbations of chronic bronchitis in patients with chronic lung disease (113, 199). Of growing concern is the increasing level of resistance to *S. pneumoniae* among commonly used antimicrobials, namely, the penicillins and other ß-lactams as well as the macrolides. Although geographically dependent, the frequencies of penicillin and macrolide resistance among strains of *S. pneumoniae* are estimated to be 40 and 30%, respectively (79, 348). Of perhaps greater concern is the rapid rate at which resistance has developed within clinical strains of *S. pneumoniae* (353). The incidence of penicillin-resistant *S. pneumoniae* increased from between 3-to-6% before 1991 to 39-to-46 % by 2000 and 2001 (31, 35, 79, 102, 348). In several studies, isolates with high-level resistance represented 13-to-20% of all strains and isolates with intermediate-level resistance represented 23-to-28% (79, 80). More recent data suggest that >70% of high-level penicillin-resistant pneumococci demonstrate cross-resistance to the macrolide class of antimicrobials (32, 79, 80).

The relatively low incidence of fluoroquinolone resistance among clinical strains of *S. pneumoniae* ($\leq$ 2% overall in Canada and the US) (337, 348, 363) has led to the use of fluoroquinolones as first-line agents for the treatment of pneumonia and lower respiratory infections both in inpatient and the outpatient settings, and particularly when antimicrobial resistance is suspected (15, 16, 154). However, fluoroquinolone-

resistance associated with clinical failures is now being reported, raising concerns over the continued use of quinolones with border-line (near breakpoint) activity against *S. pneumoniae* (32, 89, 315, 318). In Canada, Chen *et* al (53) found that the prevalence of ciprofloxacin-resistant pneumococci increased from 0% in 1993 to 1.7% in 1997-1998 (p=0.01). In adults, the prevalence increased from 0% in 1993 to 3.7% in 1998. In addition to the increase in prevalence, increases in the overall degree of fluoroquinolone resistance were also noted (53). Similar results have also been reported in Spain (211), Japan (159), Eastern Europe (254) and the United States (80). The rapid global dissemination of penicillin-resistant strains of *S. pneumoniae* (220) demonstrates the threat that antimicrobial resistance poses in the clinical setting. For the fluoroquinolone class of antimicrobials, rates of resistance to *S. pneumoniae* remain relatively low (<2%) (46), and clinicians, laboratorians, and the scientific community are faced with the daunting task of minimizing resistance while maintaining the clinical lifespan of the fluoroquinolone class of antimicrobials. This task requires a proactive approach to dealing with resistance. Unfortunately, fluoroquinolone-resistant *S. pneumoniae* are emerging (53) and, since 1999, there have been over 30 case reports of levofloxacin treatment failures in the United States and Canada (7, 70, 75, 97, 114, 186, 194, 305, 340, 351, 358).

Among the most well known and well characterized cases were those reported by Davidson *et al* (70) who documented the development of resistance mutations in strains of *S. pneumoniae* for patients receiving quinolone therapies. A report by Davies *et al* (71) involving a subset of levofloxacin-susceptible pneumococcal isolates from

the 1999-2000 respiratory season, evaluated as part of the TRUST (Tracking Resistance in the United States) study, indicated that 6.6 and 7.1% of the *S. pneumoniae* isolates for which the levofloxacin MICs are 1.0 and 2.0 μg/ml, respectively, contain a first-step *parC* mutation. Recent experiments conducted on clinical strains of *S. pneumoniae* demonstrated that of 164 unique patient isolates of *S. pneumoniae*, 29.9% harboured a mutation in either the *parC* or the *gyrA* gene, with the majority of isolates (67.3%) having a mutation in the *parC* locus only (46). An additional Canadian study which examined clinical isolates between 1993-to-1998 revealed that 59% of the isolates with a levofloxacin MIC of 2 μg/ml, a level considered susceptible by NCCLS criteria, had a first-step *parC* mutation (210). Thus, the state of fluoroquinolone efficacy for the current and future treatment of *S. pneumoniae* is clear. In order to preserve this important group of antimicrobial agents and prevent the rapid emergence of resistance, inappropriate use needs to be controlled and it is paramount that we learn which fluoroquinolones and which dosing regimes will help to minimize selection of resistant bacteria (32, 53, 164).

The MPC describes the minimal antibiotic concentration required to inhibit the most resistant first-step mutant present in large ($\geq 10^9$ CFU/ml) susceptible cultures (41, 87, 149, 367). For example, bacterial populations may reach 10^{10} cells in human infections (100, 116, 220, 241), but at a mutation frequency of 10^{-7}, more than 10^{14} bacteria ($10^7 \times 10^7$) would be required to detect two concurrent fluoroquinolone-resistant mutations (41, 367). When we examined the effect of fluoroquinolone concentration on the selection of resistant mutants of *S. pneumoniae,* we found drug

concentrations that prevented the growth of mutant colonies when 10^9-to-10^{10} cells were applied to agar plates containing antibiotic. This drug concentration, designated the MPC, would require a bacterial cell to develop more than one resistance mutation for growth in the presence of the antibiotic. Thus, at concentrations above the MPC, a bacterial population size greater than that normally present during infection would be necessary for mutant growth. Since fluoroquinolone structure affects the value of the MPC (83), we hypothesized that the MPC might serve as a simple measure of antibiotic potency that incorporates the ability of a compound to restrict selection of resistant mutants. The application of the MPC in current dosing regimes would add consideration of the development of resistance to the traditional goal of clearing infection.

Examination of large numbers of clinical isolates generally involves measurement of antibiotic potency that is typically defined by the MIC. With the agar dilution method, approximately 10^5 CFU/ml are applied to a series of agar or microtiter plates containing various antibiotic concentrations (258) and the concentration that prevents colony formation is recorded as the MIC. Measurement of MPC is carried out using the same strategy, except that more cells (approximately 10^{10} cells) are applied to agar plates containing varying drug concentrations. Consequently, it should be possible to define MPC measurements for large numbers of clinical isolates against a panel of different fluoroqouinolone agents. In the following work, I define MPC values for clinical isolates of *S. pneumoniae* against gatifloxacin, gemifloxacin, grepafloxacin, levofloxacin, moxiflxoaicn, and trovafloxacin. MPC measurements were compared

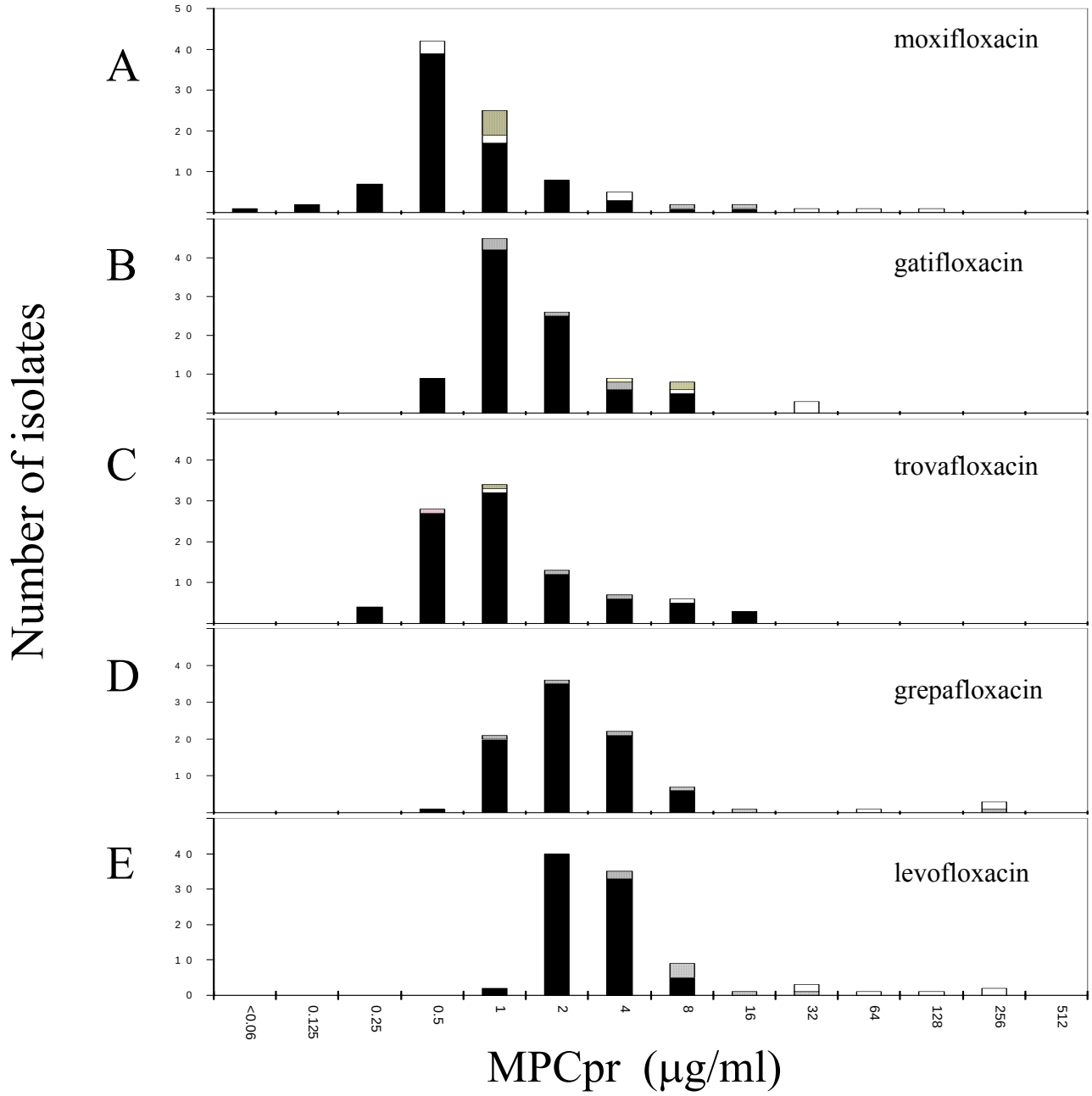
with published PK/PD parameters and bacterial killing studies were conducted to determine if, and for how long, serum/tissue drug concentrations were expected to remain in excess of the MPC for a given drug. The relationship between fluoroquinolone PK/PD and the MIC_{90}/ MPC_{90} measurements were used to empirically define the potential for a given fluoroquinolone to select for resistance.

3.3 RESULTS

3.3.1 MPC Distributions for Clinical Isolates of *S. pneumoniae*

When 10^9-to-10^{10} cells of *S. pneumoniae* were applied per plate, a sharp drop in growth was seen over a range of one-four dilutions above the MIC. When the minimal concentration which prevented the growth of mutant colonies (MPC_{pr}) was plotted against the number of isolates, distinct peaks were seen in the distribution (Figure 4.3.1). The MPC_{pr} represents a provisional value because the initial 72 hr screening process, preformed on non-drug containing media, overestimates the MPC_{90} result by 2-fold (see Materials and Methods). When the average of MPC_{pr} results for approximately 100 clinical isolates of *S. pneumoniae* were determined, the five fluoroquinolones could be ranked in terms of potency, in descending order, with moxifloxacin, > gatifloxacin = trovafloxacin >grepafloxacin > levofloxacin (Table 3.3.1). Since working with large numbers of *S. pneumoniae* isolates is cumbersome, I determined an empirical relationship between MPC and the MIC so that the MPC of a drug can be calculated from the MIC of the drug. When the MIC of a drug was measured by the agar dilution method, most of the isolates exhibited a two- to four-fold difference between the MIC and MPC_{pr} (one to three dilutions). The ratio was slightly higher for trovafloxacin and grepafloxacin. The ratio of the MPC to the MIC of a drug was calculated using values that exceeded those for 90% of the isolates (MPC_{90} and MIC_{90}). The ratio was found to be 8 for moxifloxacin, levofloxacin, and gatifloxacin, and 16 for trovafloxacin and grepafloxacin. Similar ratios were obtained for the MPC and the MIC of these drugs with the laboratory strain ATCC 49619.

Figure 3.3.1 Distribution of MPCpr Results for 5 Fluoroquinolones Against 100 Clinical Isolates of *S. pneumoniae*



White bars represent isolates containing *parC* mutations known to confer resistance; shaded regions represent isolates containing *parC* mutations that have not been demonstrated by genetic tests to confer quinolone resistance; solid regions represent unsequenced isolates.

Table 3.3.1 Fluoroquinolone Potency Based on MPCpr Results[a]

Fluoroquinolone	MPC_{pr50} (μg/ml)	MPC_{pr90} (μg/ml)	MIC_{90} (μg/ml)	MPC_{pr90} / MIC_{90}
Moxifloxacin	0.5	2	0.25	8
Trovafloxacin	1	4	0.25	16
Gatifloxacin	1	4	0. 5	8
Grepafloxacin	2	8	0.5	16
Levofloxacin	4	8	1	8

[a] From data presented in Figure 3.3.1

Figure 3.3.2 depicts MPC results obtained using drug-containing plates for the 72 hr screening process (described in Materials and Methods). Development of trovafloxacin and grepafloxacin has stopped since our initial publication on MPC (41); thus, Figure 3.3.2 illustrates MPC results for 220 clinical isolates against the currently available fluoroquinolones, (moxifloxacin, gatifloxacin, gemifloxacin, and levofloxacin) used in the treatment of infections caused by *S. pneumoniae*. Fluoroquinolone-resistant isolates, based on a moxifloxacin MIC $\geq$ 2 μg/ml, were excluded from MPC testing. Gemifloxacin had the lowest modal MPC (0.125 μg/ml), followed by moxifloxacin (0.5 μg/ml), gatifloxacin (1 μg/ml) and levofloxacin (2 μg/ml). The same rank order was observed when MPC was determined for 90% of the isolates. These data are consistent with gemifloxacin having greater *in vitro* activity than the other fluoroquinolones against resistant mutants (149, 254). When the MIC at which 90% of the susceptible isolates were inhibited (MIC_{90}) was determined, gemifloxacin was also more active than moxifloxacin, gatifloxacin and levofloxacin in these comparisons by 2, 3 and 4 doubling dilutions, respectively. Detailed descriptions of MPC results for the 220 clinical isolates are illustrated in Table 3.3.2. For moxifloxacin, 52.3% (115/220) of the isolates tested had MPC results of $\leq$ 0.5 μg/ml compared to 7.7% for gatifloxacin and 0% for levofloxacin. For gemifloxacin, 97% had a MPC of $\leq$ 0.5 μg/ml and only 1 isolate tested had a MPC result of $\geq$ 2 μg/ml. For moxifloxacin, gatifloxacin, and levofloxacin, 9%, 25.5 %, and 93% of the isolates tested

Figure 3.3.2 Distribution of MPC Results for Moxifloxacin, Levofloxacin, Gatifloxacin, and Gemifloxacin Against 220 Clinical Isolates of *S. pneumoniae*

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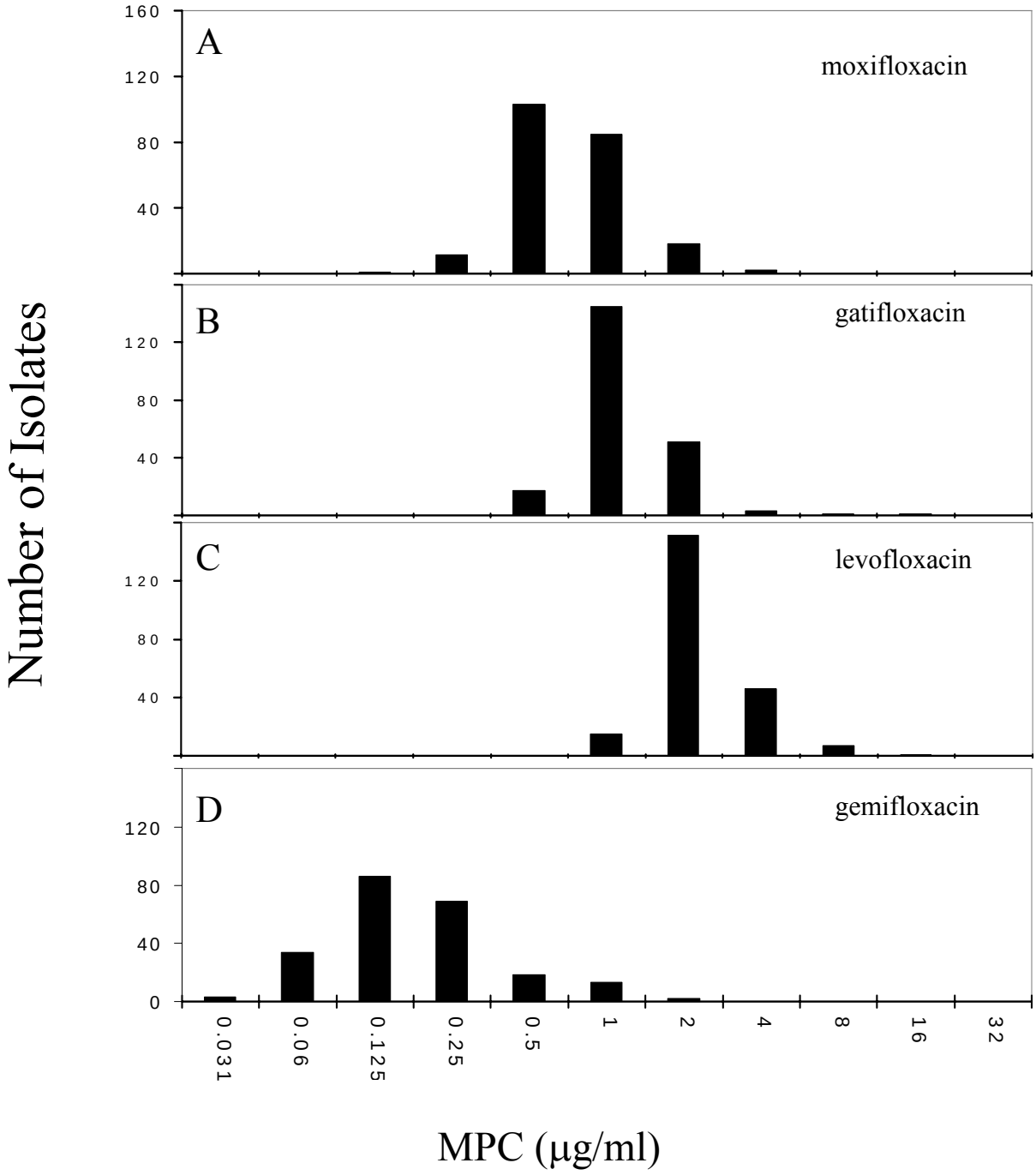


Table 3.3.2 Distribution of MPC Results and the Relationship Between MIC and MPC for Clinical Isolates of *S. pneumoniae* and Fluoroquinolones

Fluoroquinolone	No. of isolates at various MPC drug concentrations													
	≤0.031	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64		
Trovafloxacin[a] (n=100)				5	28	36	14	7	6	4				
Grepafloxacin[b] (n=98)				2	23	37	25	9	2					
Moxifloxacin (n=220)			1	11	103	85	18	2						
Gatifloxacin (n=220)					17	145	51	3	1	1				
Levofloxacin (n=220)						15	151	46	7	1				
Gemifloxacin (n=220)	3	34	86	70	14	5		1						
Fluoroquinolone	**No. of isolates having ratios of MPC/MIC[c]**													
	0.06	**0.13**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**
Trovafloxacin[a] (n=100)					1	1	28	33	22	9	4	2		
Grepafloxacin[b] (n=100)						5	18	27	31	15	3	1		
Moxifloxacin (n=220)					5	17	101	62	34		1			
Gatifloxacin (n=220)					4	68	116	29	3		4			
Levofloxacin (n=220)						118	72	8	1	1				
Gemifloxacin (n=220)					16	49	77	54	17	6	1			

[a ,b] Trovafloxacin and grepafloxacin have been withdrawn from the market. Trovafloxacin and grepafloxacin results represent provisional (MPCpr) values, which overestimate the actual MPC result 2-fold.

[c] Ratio of MPC/MIC was determined for each isolate and the number of isolates having the indicated values of the ratio were tabulated. This data set includes strains that have a known resistance mutation(s). MPC and MIC values were determined by either plating $\geq 10^9$ (MPC) or 10^5 (MIC) respectively on agar containing two-fold dilutions of fluoroquinolones.

had a MPC of $\geq$ 2 μg/ml. On average, MPC results for the six fluoroquinolones tested were 4-fold higher than MIC measurements.

3.3.2 Relationship of MPC Results to Fluoroquinolone PK/PD Properties

Since the effectiveness of an antibacterial agent is likely to be a function of both activity (MPC) and pathogen exposure, I was interested in examining the relationship between MPC results and the recommended fluoroquinolone doses and drug PK/PD parameters (Table 3.3.3) to see if compounds could be differentiated based on their potential to select for resistance during therapy. Gemifloxacin, the most potent of the compounds tested, has a C_{max} of 1.6 μg/ml with a half-life of 7-to-8 hrs. Concentrations of gemifloxacin are expected to exceed the MPC for approximately 12 hr of the dosing interval. Moxifloxacin has a maximum serum drug concentration of 4.5 μg/ml and the half-life is 12 hr. Therefore, once daily dosing should keep concentrations of moxifloxacin in serum above the MPC_{90} for the entire length of the dosing interval (i.e., 24 hr). Gatifloxacin is expected to maintain serum concentrations above the MPC_{90} for six hours and may require twice-daily dosing. Levofloxacin concentrations are expected to exceed to the MPC_{90} for 3 hr, thus, restricting the selection of resistant mutants with levofloxacin may require higher or more frequent doses.

Table 3.3.3 Fluoroquinolone Potency Based on MIC and MPC[a] Results for Clinical Isolates of *S. pneumoniae*

FQ[a]	MIC_{90} µg/ml	MPC_{50}[b] µg/ml	MPC_{90}[b] µg/ml	MPC_{90}[b] / MIC_{90}	C_{max} [e,f,g,h,I, j] (µg/ml)	Half-life (hr)	T>MPC_{50}[b] (h)	T>MPC_{90}[b] (h)	AUC_{0-24}/ MIC_{90}	AUC_{0-24}/ MPC_{90}[b]	C_{max}/ MPC_{90}[b]	C_{max}/ MIC_{90}
Trova[c] (n=100)	0.25	0.5	2	8	3.1	12	20	8	104	13	2	12
Grepa[d] (n=98)	0.5	1	4	8	2.7	14	10	0	25	2	2	5
Moxi (n=220)	0.25	0.25	1	4	4.5	12-14	>24	>24	190	48	5	18
Gati (n=220)	0.5	0.5	2	4	4.2	8-10	21.75	6	103	26	2	8
Levo (n=220)	1	1	4	4	5.7	12-14	18	3.1	48	12	1	5
Gemi (n=220)	0.06	0.125	0.5	8	1.6	7-8	16	12	140-280	16-34	3	27-53

[a]FQ= Fluoroquinolone: Trova = trovafloxacin; Grepa = grepafloxacin; Moxi = moxifloxacin; Gati = gatifloxacin; Levo = levofloxacin; Gemi = gemifloxacin. [b]MPC results were confirmed using a 72-hour drug screen. Trovafloxacin and grepafloxacin represent MPC_{90} adjusted values. Of the total 220 isolates tested, 58 were non susceptible to penicillin. Fluoroquinolone susceptibility was unaffected by loss of susceptibility to penicillin. [c, d] Trovafloxacin and grepafloxacin are not in clinical use and have been withdrawn from the market. Trovafloxacin and grepafloxacin represent MPC_{90} adjusted values. [e,f,g,h,I,j] Cmax determinations based on published 200 (trova), 800 (grepa), 400 (moxi), 500, 400 (gati), 500 (levo) and 320 (gemi) mg doses (5, 44, 69, 95, 135, 197, 222, 333, 343).

Some of the isolates examined at high inoculum concentrations required exceptionally high concentrations of fluoroquinolone to prevent colony formation (shaded bars Figure 3.3.1). Since ciprofloxacin and levofloxacin have been used extensively in Canada as therapy for respiratory tract infections including those caused by *S. pneumoniae* and since many fluoroquinolone-resistant isolates have emerged, I suspected that at least some of the isolates in the present study contained mutations in the target genes, *parC* (topoisomerase IV) and/or *gyrA* (gyrase). To test this idea, DNA was obtained from 22 isolates and the nucleotide sequences of the QRDRs of the two genes were determined. As shown in Table 3.3.4, 17 isolates were *parC* mutants, 7 of which also contained a *gyrA* mutation. Six of the *parC* mutants (isolate numbers 10, 12, 13, 15, 16 and 18) contained alleles known from genetic studies (117) to confer resistance (predicted amino acid changes of Ser-79 to Phe and Asp-83 to Asn). Of these, three also contained a GyrA alteration (Ser-81 changed to Phe or Tyr) known to confer resistance. Since MPC is based on the recovery of colonies grown from wild-type populations, the six resistant mutants (white portions of bars in Figure 3.3.1) were excluded from determination of MPC_{90} results. For 11 other isolates, the nucleotide sequence predicted changes of Ser-52 to Gly, Asn-91 to Asp, or Lys-137 to Asn in the ParC protein (shaded portion of bars in Figure 3.3.1). To my knowledge, genetic studies have not been performed that attribute resistance to these alleles. Consequently, I did not exclude these strains or those lacking a *parC* or *gyrA* mutation from the determination of the MPC_{90}.

Table 3.3.4 Fluoroquinolone-Resistance Alleles Associated With High MPC Values

Isolate no.	MPC (μg/ml)[a]						Changes in QRDR[c]	
	Gati	Grepa[b]	Levo	Moxi	Trova[b]	Gemi	*parC*	*gyrA*
wt[d]	2	4	4	1	1			
10	8	256	16	4	16		D83N (GAT to AAT)	S81F (TCC to TTC)
12	4	>256	32	4	16	2	S79F (TCT to TTT)	S81Y (TCC to TAC)
13	16	64	64		8	4	S79F (TCT to TTT) S52G (AGC to GGA) N91D (AAC to GAC)	S114G (AGT to GGT)
15	16	8	32	8	1	1	S79F (TCT to TTT)	None
16		256			16		S79F (TCT to TTT)	S81F (TCC to TTC)
18		>256			8		S79F (TCT to TTT)	None
27	8	>256	8	2	4	1	S52G (AGC to GGC) N91D (AAC to GAC)	S114G (AGT to GGT)
33	1	1	4	0.25	2	0.125	K137N (AAG to AAT)	None
35	2	4	8	0.5	4	0.5	K137N (AAG to AAT)	None
36	1	1	2	0.5	1	0.06	K137N (AAG to AAT)	None
37	1	2	2	0.5	1	0.125	K137N (AAG to AAT)	None
42	1	4	4	0.5	>16	0.125	K137N (AAG to AAT)	None
43	0.5	1	8		8	0.5	None	None
48	1	2	2	0.5	1	0.125	K137N (AAG to AAT)	None
51	0.5	2	2		1	0.06	K137N (AAG to AAT)	None
64	4	4	4	1	1	1	None	None
74	1	16	4	0.5	>8	0.25	S52G (AGC to GGC)	None
78	1	1	2	0.5	1	0.125	None	None
87	2	1	2		0.5	0.125	None	None
89	4	8	4	1	1	0.5	S52G (AGC to GGC)	S114G (AGT to GGT)
91	0.5	2	4	0.5	0.5		S52G (AGC to GGC) N91D (AAC to GAC)	S114G (AGT to GGT)
103	0.5	4	1	0.5	2	1	None	None

Gati = gatifloxacin; Grepa = grepafloxacin; Levo = levofloxacin; Moxi = moxifloxacin; Trova = trovafloxacin; and Gemi = gemifloxacin. [a] All MPC values were determined using a 72h screening process on drug containing plates, [b] MPC results obtained for grepafloxacin and trovafloxacin represent provisional values which are a two-fold overestimation of the actual MPC value. [c] QRDR, quinolone resistance determining region. Amino acid in wild-type protein is indicated before its number in the protein, followed by the amino acid change. D = aspartic acid; F = phenylalanine; G= glycine; K = Lysine; S = serine; Y = tyrosine. [d] wild type strain ATTC 49619.

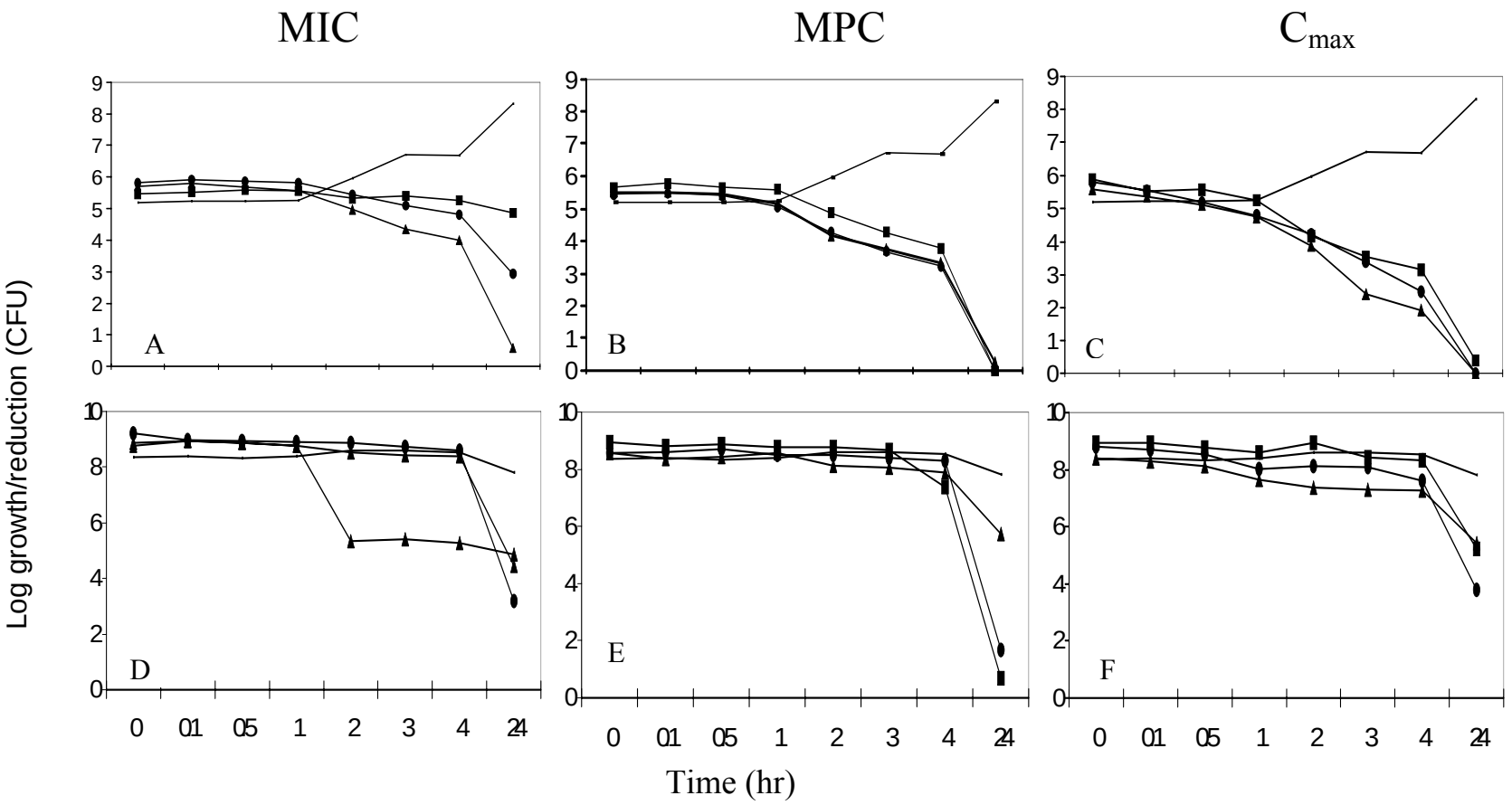
3.3.3 Killing of *S. pneumoniae* by Fluoroquinolones at the MIC, MPC and C_{max} Drug Concentrations

Like traditional measures of drug susceptibility, the MPC measurement is not reflective of bacterial killing. I was interested in examining the killing of *S. pneumoniae* by gemifloxacin, levofloxacin and moxifloxacin (by conventional kill curve methods) for four clinical strains of *S. pneumoniae* (SP-9, SP-26, SP-69, SP-73). All isolates tested were fluoroquinolone susceptible and were tested using inocula of 10^5 and 10^8 CFU/ml against the MIC, MPC, and Cmax drug concentrations. Table 3.3.5 summarizes the MICs and MPCs of the pneumococcal isolates tested in the killing experiments. Panel A in Figure 3.3.3 represents the killing of four strains exposed to MIC drug concentration for gemifloxacin, moxifloxacin, and levofloxacin using inoculums of 10^5 CFU/ml cultures. Re-growth was observed with all fluoroquinolones tested during the initial 10 min of exposure and up two one hr following exposure to levofloxacin and moxifloxacin. By 4 hr, –1.37, and - 1.0 log reductions in viable counts were measured for gemifloxacin and moxifloxacin, and a - 0.06 log reduction in viable counts was measured for levofloxacin (table 3.3.6). After 24 hr, - 5.13, - 2.87, and - 0.58 log reductions in viable counts were measured for gemifloxacin, moxifloxacin, and levofloxacin respectively. A 73% reduction in viable cells was calculated for levofloxacin after 24 hr compared to > 99% for gemifloxacin and moxifloxacin. When 10^8 CFU/ml cultures were tested against the MIC drug concentrations, the log reductions in viable counts for gemifloxacin, moxifloxacin and levofloxacin over 4 hr were - 0.34, - 0.48, and - 3.62.

Table 3.3.5 MIC and MPC Results for Moxifloxacin, Gemifloxacin, and Levofloxacin Against 4 Isolates of *S. pneumoniae*

Isolate ID	Susceptibility Test					
	Moxifloxacin		Gemifloxacin		Levofloxacin	
	MIC (μg/ml)	MPC (μg/ml)	MIC (μg/ml)	MPC (μg/ml)	MIC (μg/ml)	MPC (μg/ml)
Sp9	0.125	0.5	0.03	0.125	0.5	2
Sp26	0.125	0.5	0.06	0.25	1	4
Sp69	0.125	0.5	0.03	0.125	1	2
Sp73	0.125	1	0.03	0.25	0.5	2

Figure 3.3.3 Killing of *S. pneumoniae* (n=4) by Gemifloxacin, Moxifloxacin, and Levofloxacin at the MIC, MPC and C_{max} Drug Concentrations



Four strains of S. pneumoniae were tested by gemifloxacin (triangles), moxifloxacin (circles) and levofloxacin (squares). The *S. pneumoniae* ATTC 46919 growth control (incubated in the absence of drug) is represented by a blank line. Graphs depict the average inocula tested and the average killing for the four strains tested. Panels A, C, and E represent killing of 10^5 CFU/ml cultures. Panels B, D, and F depict killing of 10^8 CFU/ml cultures.

Table 3.3.6 Killing of *S. pneumoniae* (n=4) at Varying Concentrations and Inocula for Clinical Isolates of *S. pneumoniae*

Time (hr)	Measurement	Drug Concentration Tested in Kill Experiments (inoculums tested (CFU/ml)								
		MIC			MPC			C_{max}		
		Gemi ($3.89x10^5$)	Levo ($7.81x10^5$)	Moxi ($6.17x10^5$)	Gemi ($3.89x10^5$)	Levo ($7.81x10^5$)	Moxi ($6.17x10^5$)	Gemi ($3.89x10^5$)	Levo ($7.81x10^5$)	Moxi ($6.17x10^5$)
1	% reduction/re-growth in viable cells	-30.80	+26.60	+2.30	-56.60	-12.90	-58.30	-85.10	-76.40	-90.20
3		-95.70	-12.90	-80.40	-98.30	-95.60	-96.70	-99.90	-99.64	-99.60
4		-95.70	-36.90	-90.20	-99.35	-98.64	-99.44	-99.94	-99.82	-99.61
24		>-99.99	-73.89	-99.80	>-99.99	-100	-100	-100	>-99.99	-100
1	Log reduction/re-growth	-0.16	+0.10	+0.14	0.00	-0.06	-0.43	-0.86	-0.63	-1.01
3		-0.76	-0.13	-0.38	-1.77	-1.36	-1.80	-3.19	-2.38	-2.41
4		-1.37	-0.06	-1.01	-2.19	-1.87	-2.25	-3.68	-2.75	-3.30
24		-5.13	-0.58	-2.87	-5.24	-5.65	-5.49	-5.59	-5.50	-5.78
Time (hr)	Measurement	MIC			MPC			C_{max}		
		Gemi ($2.51x10^8$)	Levo ($8.81x10^8$	Moxi ($6.57x10^8$)	Gemi ($2.51x10^8$)	Levo ($8.81x10^8$)	Moxi ($6.57x10^8$)	Gemi ($2.51x10^8$)	Levo ($8.81x10^8$)	Moxi ($6.57x10^8$)
1	% reduction/re-growth in viable cells	-20.1	-17.8	-50.7	+2.9	-32.4	-84.5	-41.49	-52.29	-84.59
3		-63.5	-13.9	-66.8	-67.4	-48.7	-81.9	-62.89	-70.19	-81.99
4		-59.50	-99.97	-76.01	-78.62	97.31	-49.30	-92.76	-75.59	-93.65
24		>-99.99	-99.89	>-99.99	>-99.99	>-99.99	>-99.99	-99.77	-99.99	>-99.99
1	Log reduction/re-growth	+0.01	-0.09	-0.82	-0.01	-0.17	0.00	-0.76	-0.33	-0.81
3		-0.24	-3.55	-0.61	-0.49	-0.35	-0.18	-1.08	-0.53	-0.74
4		-0.34	-3.62	-0.48	-0.67	-1.57	-0.30	-1.14	-0.63	-1.20
24		-4.34	-4.00	-6.03	-2.81	-8.25	-6.92	-2.97	-3.73	-5.03

[a]MIC=minimum inhibitory concentration; MPC=mutant prevention concentration; C_{max}=maximum serum concentration; Gemi-gemifloxacin; levo=levofloxacin; moxi=moxifloxacin.

respectively. By 24 hr, - 4.34, - 6.03, and - 4.00 log reductions in viable counts were measured for gemifloxacin, moxifloxacin, and levofloxacin, respectively, which represented > 99% killing for all three fluoroquinolones tested. At the MPC drug concentrations (panel C), - 2.19, - 2.25, and - 1.87 log reductions in viable counts were measured after 4 hrs and killing of > 98% was observed for all three fluoroquinolones tested. After 24 hr, > - 5 log reductions in viable counts were measured for all three fluoroquinolones tested. When 10^8 CFU/ml cultures were exposed to the MPC drug concentrations (panel D) log reductions in viable cells were measured at - 0.67, - 0.30, and - 1.57 for gemifloxacin, moxifloxacin, and levofloxacin, respectively. These values increased to - 2.9, - 6.9 and - 8.25 respectively after 24 hr and resulted in $\geq$ 99.99% killing for all three fluoroquinolones.

When 10^5 CFU/ml cultures were exposed to the Cmax drug concentration, a $\geq$-3 .0 log reduction was observed after 4 hr for gemifloxacin, levofloxacin and moxifloxacin. After 24 hrs of exposure, - 5.6, - 5.5 and – 5.6 log reductions in viable counts were observed for gemifloxacin, levofloxacin and moxifloxacin, respectively. No growth was observed for gemifloxacin and moxifloxacin when 10^5 CFU/ml cultures of *S. pneumoniae* were exposed to the C_{max}. The killing of 10^8 cultures at the Cmax was less than that observed with 10^6 CFU/ml cultures. After 4 hours, - 1.1, - 0.6 and - 1.1 log reductions in growth were seen for gemifloxacin, levofloxacin and moxifloxacin, respectively. By 24 hr, killing of $\geq$ 2.0 log was observed for all agents tested; - 2.0, - 3.1 and - 3.8 log reductions in viable counts were observed for gemifloxacin,

levofloxacin and moxifloxacin, respectively, which resulted in $\geq$ 99.78% killing for all three fluoroquinolones.

3.4 DISCUSSION

Preliminary MPC work with *S. pneumoniae* (40) revealed a narrow drug concentration range for mutant selection and led me to expect that agar plates used in a standard two-fold dilution analysis with clinical isolates would exhibit either confluent growth or no colonies, at least for the C-8-methoxy compounds. When 10^9-to-10^{10} cells were applied per plate, a sharp drop in growth was seen over a concentration range of one to four dilutions above the MIC. When the minimal concentration at which no colonies were recovered (MPC_{pr}) was plotted against the number of isolates, distinct peaks were seen in the distribution (Figure 3.3.1). The peak for moxifloxacin appeared at the lowest drug concentration; therefore, moxifloxacin was the most potent fluoroquinolone by this assay. I consider the values of MPC_{pr} shown in Figure 3.3.1 to be provisional because initial 72 hr screens used to confirm the absence of growth, were preformed on non-drug containing plates. When plates with antibiotic concentrations at the MPC were used to confirm the absence of growth, it was determined that MPC_{pr} results were a 2-fold overestimation of the MPC. The observed difference between MPC_{pr} and the actual MPC results appear to be due to differences in bacteriostatic versus bactericidal activity. A heavy bacterial suspension containing a mixture of cells and capsular polysaccharides may have resulted in a thin "film" on some of the plates which in turn may have provided a "protective effect" when the contents of the plate were transferred to non-drug containing media and re-incubated for an additional 24 hr. These data also argue against complications due to autolysis occurring at high cell

density, since that would have made MPC_{pr} an underestimation of MPC. In a separate experiment using a laboratory isolate of *S. pneumoniae* (ATCC 49619), various fluoroquinolone concentrations were tested with dilute bacterial cultures and many agar plates so that mutants were recovered as single colonies (41). The MPC for the ATTC 49619 strain was 0.5 μg/ml for moxifloxacin and 2.6 μg/ml for levofloxacin. The MPC_{pr} was 1 μg/ml for moxifloxacin and 4 μg/ml for levofloxacin using the 2-fold agar dilution assay described in Materials and Methods. Consequently, MPC_{pr} overestimates MPC by an average of 2- fold for both the most active and the least active compounds in the present study.

When the average of MPC_{pr} results for approximately 100 clinical isolates of *S. pneumoniae* were determined, the five fluoroquinolones could be ranked in terms of potency, in descending order, with moxifloxacin, > gatifloxacin = trovafloxacin >grepafloxacin > levofloxacin (Table 3.3.1). Based on MPC results, intrinsic differences in the anti-pneumococcal activity for the agents tested were observed and in many cases, these differences were not reflected in the MIC measurements of susceptibility. When the MICs and MPCs were determined using the same set of isolates for each compound, I found that gemifloxacin had the lowest modal MPC (0.125 μg/ml), followed by moxifloxacin (0.5 μg/ml), gatifloxacin (1 μg/ml) and levofloxacin (2 μg/ml). The same rank order was observed when MPCs were determined for 90% of the isolates. These data are consistent with gemifloxacin having superior *in vitro* activity when compared to other quinolones (254, 340). When the

MIC at which 90% of the susceptible isolates are inhibited (MIC_{90}) was determined, gemifloxacin was also more active than moxifloxacin, gatifloxacin and levofloxacin in these comparisons by 2, 3 and 4 doubling dilutions, respectively.

Since working with large numbers of *S. pneumoniae* isolates is cumbersome, I determined an empirical relationship between MPC and MIC so that the MPC of a drug can be calculated from the MIC of the drug. When the MIC of a drug was measured by the agar dilution method, most of the isolates exhibited a 2- to 4-fold difference between the MIC and MPC_{pr} for five fluoroquinolones tested. The ratio was slightly higher for trovafloxacin and grepafloxacin. The ratio of the MPC to the MIC of a drug was calculated using values that exceeded those for 90% of the isolates (MPC_{90} and MIC_{90}); this ratio was 8 for three of the compounds and 16 for trovafloxacin and grepafloxacin. Similar ratios were obtained for the MPC and the MIC of these drugs with the laboratory strain ATCC 49619. Large numbers of isolates tested against six different quinolone agents provides support for the extrapolation of MPC value from the MIC for strains of *S. pneumoniae*, provided few strains with MICs $\geq$ 2 μg/ml to moxifloxacin are present in the population. Ideally, a small MPC/MIC ratio should be targeted and would correlate with a narrow mutant selection window, since the time drug concentrations are in above the MIC and below the MPC is expected to be small. Increased incidence of fluoroquinolone-resistant *S. pneumoniae* have been associated with treatment of penicillin-resistant cases of pneumonia (53). However, for the newer fluoroquinolones (such as moxifloxacin and gemifloxacin), the value of MIC_{90} is

unaffected by pneumococcal resistance to penicillin and/or other agents (27, 31, 33). Likewise, no increase in MPC was associated with the presence of penicillin resistance in the present data set. Thus, potential cross-resistance between the two classes of compounds does not appear to be factor in quinolone-resistance.

Sequence analysis of the QRDR of *S. pneumoniae* isolates with high MPCs revealed the presence of first-step *parC* mutations and double *parC* + *gyrA* mutations. While susceptible isolates that harbour a *parC* mutation lead to higher MPC results, the significance of these isolates in the general population remains unknown, in part because we are currently unable to detect them. Apart from DNA sequencing, no accurate test can reliably identify isolates with first-step mutations (210). In the context of isolates with pre-existing *parC* mutations, the value of the MPC measurement will become important, because it will be able to identify isolates which are difficult to treat isolates. Recent fluoroquinolone-treatment failures in *S. pneumoniae* have been associated with strains that harboured first-step *parC* mutations prior to receiving treatment (7, 70). *In vitro* studies have shown that upon acquisition of a first-step mutation, the likelihood of developing a subsequent mutation is enhanced in comparison to the development of the first-step mutation itself (128, 207, 282). Studies preformed with *E. coli* have shown that a *parC* resistance allele, which has no effect on the MIC of the drugs, can increase by orders of magnitude the frequency at which resistant mutants are selected by C-8-methoxy fluoroquinolones (369). Therefore, minimizing the number of strains in the population which contain first-step mutations

and restricting their growth are key factors in helping to minimize fluoroquinolone resistance.

A central question surrounding fluoroquinolone resistance is whether the choice of fluoroquinolone will influence the selection of first-step mutations and the development of resistance. Less active compounds based on higher MPCs are more likely to select for resistant mutants during therapy if MPC concentrations are not maintained throughout dosing. Thus, I expect continued use of ciprofloxacin and levofloxacin to seriously shorten the useful lifespan of moxifloxacin, gatifloxacin, and gemifloxacin. Although the latter two compounds may be potent enough to treat infection caused by *parC* mutants (selected by ciprofloxacin and levofloxacin), *parC gyrA* double mutants would be readily selected by newer generation fluoroquinolones if the pathogens already contain a *parC* resistance allele. Thus, slowing the development of resistance may necessitate careful management of compounds within the same general class (41).

The endpoint of antibiotic dosing strategies, such as those based on area under the inhibitory concentration curve, has often employed clearance of infection measured either as survival in animal models or loss of symptoms in humans. Widespread development of resistance suggests that consideration should also be given to restricting the selection of resistant mutants. One approach has utilized the ratio of maximum concentration of drug in serum to the MIC of the drugs andsubsequent values have been found that restrict mutant outgrowth *in vitro* (25) and correlate with animal survival

(93). However, this ratio does not take into account the time for which drug concentrations remain high and which may be important for preventing development of resistance. Consequently, I have examined the relationship of the MPC to fluoroquinolone pharmacokinetic properties.

Compounds and dosing protocols can readily be compared for the time that drug concentrations in tissue/serum exceed the MPC. I have previously suggested that for the MPC to be a therapeutically useful parameter, its value must be below serum and tissue drug concentrations attained following administration of drug doses that are safe for patients (41, 149). Gemifloxacin, the most potent of the compounds tested, has a C_{max} of 1.6 μg/ml with a half-life of 7-to-8 hr. Concentrations of gemifloxacin are expected to exceed the MPC for approximately 12 hr of the dosing interval. Moxifloxacin has a maximum serum drug concentration of 4.5 μg/ml and the half-life is 12 hr. As such, once daily dosing should keep concentrations of moxifloxacin in serum above the MPC_{90} for the entire length of the dosing interval (i.e., 24 hr) and minimize the potential that resistant mutant will be selected during therapy. Gatifloxacin may also restrict the selection of resistant mutants, especially if administered twice daily. Levofloxacin may require higher doses, perhaps administered two to three times daily, to attain the same potency with respect to restricting selection of resistant mutants.

Examination of the concentrations present at sites of infection and in infected tissues will help to further define drug concentrations which inhibit first-step mutants and since quinolone concentrations in relevant tissue may be higher than in serum (23,

162), dosing based on the MPC may serve as a realistic approach for minimizing resistance and maximizing therapeutic outcome. Animal experiments and clinical trials are now required to test these hypotheses. The serum drug concentrations listed in Table 3.4.1 represent total concentrations. Fluoroquinolones bind to serum proteins, so additional corrections may be necessary when particular compounds are compared. Protein binding varies among the compounds, but in general less than half of the total drug that is present is bound and consequently, protein binding probably has little effect on the conclusions reached above. The time serum/tissue concentrations exceed the MPC (T > MPC) will directly correlate with reduced likelihood of selecting resistant mutants because concentrations required to inhibit their growth (and possibly kill) will be maintained throughout dosing. For fluoroquinolones, the Cmax/MIC (or Cmax/MPC) ratio has been suggested as the most important PK property associated with bacterial eradication (94). Thus, it could be perceived that that time above the MIC/MPC is not the most useful PK parameter used to assess fluoroquinolone potency. Recently, Shenteg *et al* (316, 319) summarized PK/PD data relating to clinical trials, animal studies, and *in vitro* models with fluoroquinolones and concluded that AUIC values of >100 are predictive of clinical and microbiologic outcomes and reduced incidence of resistance for fluoroquinolones. Like the T > MPC relationship, the AUIC takes into account the length of time a pathogen is exposed to inhibitory concentrations throughout a pharmacokinetic profile. Therefore, like the AUC, the T > MPC

Table 3.4.1 PK/PD Properties for Fluoroquinolones

Agent	**Dose (mg)**	**Cmax (μg/ml)**	**Tmax (hr)**	**$t_{1/2}$ (hr)**	**AUC (mg x hr/l)**	**Cmax/MPC**	**% Protein binding**	**Ref**
Levofloxacin	500	5.2-6.2	1.7	6.3-7.4	47.7	1.3-1.55	26-31	(8, 55-57, 68, 111, 135, 364)
	750	7.13-11			82	1.8-2.8		
	500 (I.V)	6.4			48.3	1.6		
	750 (I.V)	12.1			110	3		
Gatifloxacin	200	1.71	1.5	8.4	31.7-33	0.9	20	(28, 91, 257, 355, 364)
	400	3.4-4.2				1.7-2.1		
Gemifloxacin	320	1.19-1.5	1-2	8	9.3	2.38-3	58	(5, 364)
Moxifloxacin	400	4.5	0.8-2	12	48-200	4.5	37	(33, 68, 333, 364)

relationship does not contradict concentration dependent killing by fluoroquinolones, but may in fact complement it (32). Fluoroquinolones (such as moxifloxacin) that maintain serum/tissue concentrations in excess of the MPC throughout the dosing interval are expected to restrict the selection of resistant mutants.

Direct comparisons of fluoroquinolones based on the MPC requires examination of the MPC result in relation to resistance breakpoints, drug PK, the type(s) of mutants selected, and the clinical history. For example, comparison of moxifloxacin and levofloxacin revealed that 9% of the isolates tested with moxifloxacin had a MPC value at or above an intermediate breakpoint (i.e., $\geq$ 2 μg/ml), compared to 25% of the isolates tested against levofloxacin, which had MPC values at or above an intermediate breakpoint ($\geq$ 4 μg/ml) for resistance. Thus despite the fact that a larger fraction of isolates tested against levofloxacin had a lower MPC/MIC ratio than was seen for moxifloxacin, a larger number of isolates tested against levofloxacin had a MPC value that would be considered intermediate or above by conventional susceptibility guidelines. The resistance breakpoints established by the NCCLS serve as a surrogate marker of whether therapy is likely to be successful, and strains displaying intermediate and/or resistant phenotypes have typically been associated with clinical failures and the development of resistance (7, 70, 75, 351). The larger numbers of levofloxacin MPCs that fell within an intermediate breakpoint are of particular concern in light of recent evidence demonstrating that over half of the *S. pneumoniae* population with a levofloxacin MIC of 2 μg/ml, may contain a first-step mutation in *parC*. The

requirement of first-step mutations in the subsequent development of high-level fluoroquinolone resistance has been well documented (164, 207, 210, 275, 370) and supports the hypothesis that inhibiting first-step resistant mutants with MPC drug concentrations will severely restrict the selection of fluoroquinolone resistant strains. Thus, the significance of the MPC results should be interpreted in relation to: 1.) The pharmacokinetics dose-response profile of the drug. 2.) The relationship between the MPC and the susceptibility breakpoint for resistance. 3.) The types of mutants selected, and, 4.) the overall potency of the agent. These factors are likely to differ among agents within the fluoroquinolone class of antimicrobials.

Overall, the MPC measurement is a useful tool in determining appropriate quinolone therapy for the treatment of infections caused by *S. pneumoniae.* First-step resistant mutants of *S. pneumoniae* were selected from susceptible cultures when $\geq 10^9$ cells were applied to agar-containing plates seeded with increasing concentrations of quinolones. In all cases, the MPC measurement was in excess of the MICs of the wild-type cultures. Fluoroquinolones that possess low MPC values, maintain serum/tissue concentrations above the MPC_{90} for the entire length of a dosing interval, maintain a narrow MPC/MIC relationship and possess bactericidal activity against first-step resistant mutants are good candidates for restricting the selection of fluoroquinolone-resistant *S. pneumoniae.* Based on this criterion, moxifloxacin and gemifloxacin are likely to severely restrict the development of resistant mutants if the principles of MPC are applied during dosing. Gatifloxacin may require twice daily dosing in order to

restrict the development of resistant mutants. Levofloxacin had the highest MPCs of all the fluoroquinolones tested, and may require additional and/or higher doses to restrict the selection of resistant mutants of *S. pneumoniae.*

4.0 ENRICHMENT OF FLUOROQUINOLONE-RESISTANT MUTANT SUBPOPULATIONS OF *P. AERUGINOSA*

4.1 Abstract

Ciprofloxacin and levofloxacin are potent first-line fluoroquinolones which are often used in combination with anti-pseudomonal cephalosporins and/or aminoglycosides, for the treatment of infections caused by *P. aeruginosa.* However, microbiological factors (MIC, MBC, killing) and PK considerations such as the C_{max}/MIC and AUC, have not always correlated with the clinical outcome and development of resistance in cases of infections caused by *P. aeruginosa.* In an attempt to examine unresolved issues related to selection of resistant mutants of *P. aeruginosa,* I measured MPC values for 155 clinical isolates of *P. aeruginosa* for ciprofloxacin and levofloxacin. The MPC_{90} values for ciprofloxacin and levofloxacin were 4 and 16 μg/ml, respectively, suggesting that neither ciprofloxacin nor levofloxacin is suitable as monotherapy. Based on currently administered I.V. doses, ciprofloxacin is projected to remain in excess of the MPC_{90} for 5.5 hr while no currently recommended dose of levofloxacin is expected to exceed the MPC_{90} value. Levofloxacin was $\geq$ 2-fold less active against first-step resistant mutants of *P. aeruginosa* compared to ciprofloxacin, and for strains over expressing four different efflux mechanisms, levofloxacin was an average of 3 times less active than ciprofloxacin. When mutant selection was examined for six susceptible strains of *P. aeruginosa,* a larger proportion of the mutant population was recovered when tested against levofloxacin. DNA sequence analysis of selected

mutants revealed alterations in the *gyrA* and *parC* genes. Killing experiments preformed at the MPC and MIC drug concentrations for 10^6 and 10^{10} CFU/ml cultures demonstrated that maintaining MPC drug concentrations severely restricts the selection of resistant mutants. Preliminary results from a rat abscess model of infection revealed that resistant mutants are readily selected when ciprofloxacin and levofloxacin were dosed once a day. Collectively, MPC experiments conducted with ciprofloxaicn and levofloxacin against clinical isolates of *P. aeruginosa* provide a microbiological basis for observed clinical differences between ciprofloxacin and levofloxacin against *P. aeruginosa* and support the hypothesis that superior clinical performance of ciprofloxacin correlates with activity against resistant mutant subpopulations measured *in vitro*.

4.2 INTRODUCTION

Opportunistic infections caused by *P. aeruginosa* are a major cause of morbidity and mortality in nosocomial settings as well as in patients with significant underlying disease (150). Such infections may occur at any anatomical site, but frequently they are located in the respiratory and urinary tracts (125). The frequency with which *P. aeruginosa* causes disease is reliably estimated from annual surveillance data. *P. aeruginosa* was the second most common cause of nosocomial pneumonia (14% of cases), the third most common cause of urinary tract infection (7%), the fourth most common cause of surgical site infection (8%), the seventh most frequently isolated pathogen from the bloodstream (2%) and the fifth most common isolate (9%) overall from all sites (1, 50, 174, 178). Intrinsic resistance of *P. aeruginos*a to a wide variety of antibiotics has made treatment of these infections difficult, often requiring combination therapies and only a limited number of antibiotic agents retain reliable activity against this patahogen, including aminoglycosides, anti-pseudomonal penicillins, carbapenems, cephalosporins, and fluoroquinolones (124, 125, 127). Fluoroquinolones are among the more useful agents because they possess good antibacterial activity, oral bioavailabity, tissue penetration and sustainable drug concentrations (9, 28, 132, 364). However, in some instances, clinical success has sometimes been only modest and failure is often associated with the presence of resistant organisms (108, 228, 238, 280) .

Recent reports have documented declining susceptibility of ciprofloxacin and other contemporary fluoroquinolones when tested by commercial or reference methods

against clinical isolates of *P. aeruginosa* (183). For example, among 404 *P. aeruginosa* strains isolated in 2000, resistance rates were 20.5, 20.3 and 23.0% for ciprofloxacin, levofloxacin and gatifloxacin, respectively, (183) and were higher than those observed for ceftazidime, gentamicin, imipenem and piperacillin/tazobactam (susceptibility range, 82.9-to-89.9%). Sahm *et al* (310) reported 21.6-to-24.9% resistance to ciprofloxacin and levofloxacin, respectively, against isolates of *P. aeruginosa* collected by two surveillance networks during 1999-to-2000. An additional study reported a 10% decrease in fluoroquinolone susceptibility among isolates of *P. aeruginosa* from 1994-to-2000 (259). Globally, fluoroquinolone resistance rates to *P. aeruginosa* range from 12-to-75% (76, 339, 344), however, because of a lack of effective alternative agents, fluoroquinolones remain first-line agents for the treatment of severe pseudomonal infections (106, 124-127, 178, 262). Therefore, retaining and protecting fluoroquinolone activity against *P. aeruginosa* remains important.

Fluoroquinolone treatment of infection caused by *P. aeruginosa* may represent a situation in which clinical resistance reflects activity mutant sub-populations. Two commonly used compounds, ciprofloxacin and levofloxacin, are not readily distinguished by traditional measures: ciprofloxacin has greater intrinsic activity and is better able to block pathogen growth as indicated by a 2-fold lower MIC, while levofloxacin is more lethal at equivalent concentrations and achieves higher serum/tissue concentrations (225, 323). The debate over fluoroquinolone potency

against *P. aeruginosa* is exemplified in a recent report by Jones *et al* (177) who documented the contemporary equality in fluoroquinolone activity against *P. aeruginosa* based on overall rates of fluoroquinolone resistance (177). Conventional wisdom suggests that differences in intrinsic activities, bacterial killing, and drug PK, serve to normalize anti-pseudomonal activities of ciprofloxacin and levofloxacin, however, several clinical observations suggest that preferential use of specific agents may escalate the rate of quinolone-resistant *P. aeruginosa* (22, 283, 303).

Historically, ciprofloxacin has been the fluoroquinolone of choice for treating pseudomonal infections because it has the highest intrinsic activity (36), but pricing differences between ciprofloxacin and ofloxacin/levofloxacin prompted some hospital centres to switch to ofloxacin and, subsequently levofloxacin for the treatment of infections caused by *P. aeruginosa*. Peterson *et al* (283) discovered that a change from ciprofloxacin to ofloxacin use correlated with a 20% increase in fluoroquinolone resistance that was partially reversed when ciprofloxacin use was reinstated. Rifenberg *et al* (303) investigated the influence of fluoroquinolone purchasing patterns on antimicrobial expenditures and *P. aeruginosa* susceptibility. As part of this study, 109 hospitals were followed over a 4-year period. *P. aeruginosa* susceptibilities were compared between hospitals that had ciprofloxacin or ofloxacin on their formularies and did not change to another quinolone as compared to those institutions that switched from ciprofloxacin to ofloxacin. A 5.4% decrease in overall fluoroquinolone susceptibility correlated with a $300.00 (US dollars) increase in ofloxacin expenditure

per occupied bed. No associations were found between increased ciprofloxacin expenditures and subsequent changes in *P. aeruginosa* susceptibility to quinolones (303). A subsequent follow up study, which included data collected for levofloxacin, confirmed a 5% increase in quinolone resistance which occurred for every $300 (US dollars) increase in levofloxacin expenditure per occupied bed which was not observed for ciprofloxacin (22). The question of whether a change in quinolone use could influence class resistance was again raised by Pangrazzi *et al* (279) who observed decreased activity of fluoroquinolones to *P. aeruginosa* (78% to70%) in one hospital following an eight month conversion from ciprofloxacin to levofloxacin. While this data suggests the switch to levofloxacin may have impacted *P. aeruginosa* susceptibility to fluoroquinolones, this study is limited by the fact that observations occurred over a relatively short time frame (8 months) and no statistical significance was identified. Polk *et al* (289, 290) reported on trends in fluoroquinolone prescribing from 35 US hospitals in relation to fluoroquinolone resistance in *P. aeruginosa.* Total fluoroquinolone use remained stable over two years, however, levofloxacin use increased significantly while ciprofloxacin use decreased slightly. Resistance of *P. aeruginosa* to quinolones increased in 22 of the 35 hospitals and a significant positive relationship between total fluoroquinolone use and resistance to *P. aeruginosa* was seen ($r=0.54$, $p=0.01$). Mohr *et al* (243) studied the impact of antimicrobial usage on susceptibility of *P. aeruginosa* over an eight year period at a community-based 700 bed teaching hospital. The study period ranged from 1995-to-2002 and a statistically

significant correlation was seen between 4th generation cephalosporin (r^2=0.782, p=0.004) or levofloxacin use (r^2=0.726, p=0.007) and the development of quinolone-resistant *P. aeruginosa*. No such relationship existed for other antimicrobial agents investigated which included ciprofloxacin, suggesting that the development of fluoroquinolone resistance is associated with the use of individual agents rather than overall antibiotic consumption.

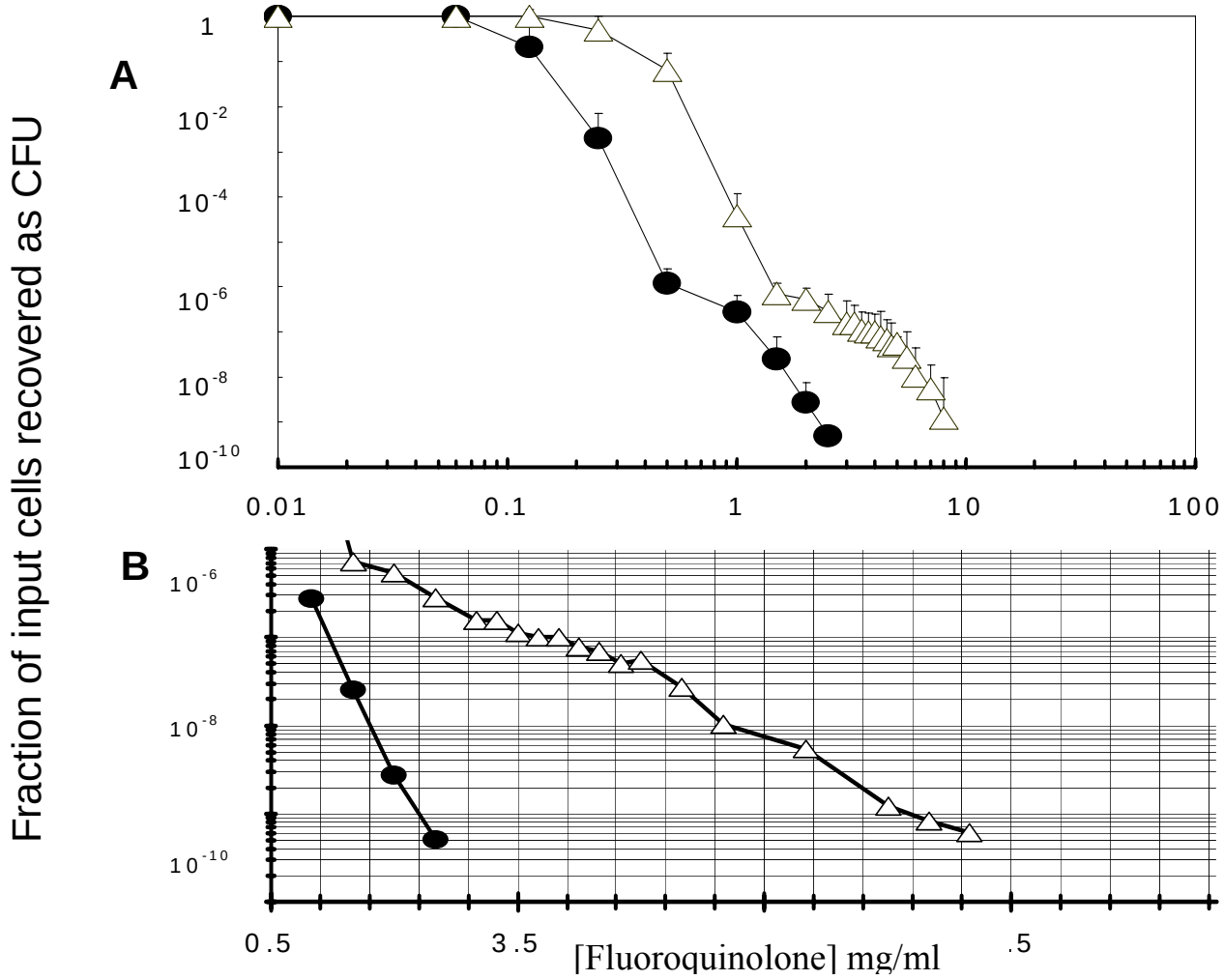
These findings raise the question of whether differences in fluoroquinolone resistance could be attributed to undefined microbiological properties of ciprofloxacin and levofloxacin. The MPC is a measure of the selection potential for resistance for any given fluoroquinolone-pathogen combination and is defined by the lowest antimicrobial concentration required to inhibit the most resistant first-step resistant mutant present in large, overall susceptible bacterial populations. Threshold conditions for restricting mutant growth have been defined *in vitro* (86) and in mice (179), however, few clear relationships have been established between clinical performance and *in vitro*, anti-mutant activity. MPC results for ciprofloxacin and levofloxacin for clinical isolates of *P. aeruginosa* were examined and compared in an attempt to identify possible differences between the ability of the antimicrobial agents to prevent emergence of antimicrobial resistance.

4.3 RESULTS

4.3.1 Effect of Fluoroquinolone Concentration on Mutant Recovery for Fluoroquinolone-Susceptible Clinical Isolates of *P. aeruginosa.*

The recovery of colonies from 10^{10} CFU/ml cultures of fluoroquinolone-susceptible clinical isolates of *P. aeruginosa (*n=6*)* tested against increasing concentrations of ciprofloxacin or levofloxacin resulted in a two-stage decline in CFUs (Figure 4.3.1). Low to moderate fluoroquinolone concentrations ($\leq$ 0.5 μg/ml) produced a gradual decline in CFUs of *P. aeruginosa* when tested against ciprofloxacin and levofloxacin, respectively. The MPC measurement can be characterized as a characteristic two-stage decline in CFUs when 10^{10} cells/ml were applied to agar plates containing increasing concentrations of fluoroquinolones. The initial MIC, MPC, and numbers of cells tested in each experiment are listed in Table 4.3.1. The first stage in colony decline results from the decline of susceptible cells and occurred at fluoroquinolone concentrations that approximated the MIC of the wild-type strains (ciprofloxacin MIC = 0.21 μg/ml, levofloxacin MIC = 0.7 μg/ml). At fluoroquinolone concentrations above the MIC of the wild-type strains, mutant growth could be measured via a generalized plateau region in colony recovery. Increasing fluoroquinolone concentrations resulted in a second decline in CFUs until a concentration of ciprofloxacin and levofloxacin was reached which prevented the growth of the most-resistant cell present in 10^{10} CFU/ml cultures. This concentration defined the MPC for each fluoroquinolone and occurred at 3 and 9 μg/ml for ciprofloxacin and levofloxacin, respectively. Comparison of the anti-mutant

Figure 4.3.1 Effect of Fluoroquinolone Concentration on Recovery of *P. aeruginosa* Colonies from Six Fluoroquinolone-Susceptible Isolates



Clinical isolates were applied to TSA plates containing the indicated concentrations of ciprofloxacin (filled circle) or levofloxacin (open triangle). The fraction of input cells recovered as mutants was determined after re-growth of colonies on drug-free agar followed by regrowth on agar containing the fluoroquinolone at the initial drug selection concentration. Each data point reflects the average decline in CFUs from six susceptible isolates of *P. aeruginosa* when 10^{10} cells/ml were tested aginst defined ciprofloxacin and levofloxacin concentrations. Panel A. Average values for seven susceptible clinical isolates (ciprofloxacin MIC = 0.21 μg/ml; levofloxacin MIC = 0.7 μg/ml). Panel B represents the recovery of resistant mutants as a linear function of fluoroquinolone concentration. Differences in mutant recovery between ciprofloxacin and levofloxacin were determined based on the area for which mutant selection occurred which is expressed in a ciprofloxacin:levofloxacin ratio of 1:1500. Error bars represent the standard deviation in the measurements for the six strains testd

Table 4.3.1 MIC and MPC Results for Clinical Isolates of *P. aeruginosa* Used in Mutant Selection Curves

Isolate	Source	MIC[a] measurement					MPC measurement			
		Ciprofloxacin		Levofloxacin		Susceptibility Result[b]	Ciprofloxacin		Levofloxacin	
		# of cells Tested (CFU)	MIC (μg/ml)	# of cells tested	MIC (μg/ml)		# of cells Tested (CFU/0.3ml)	MPC (μg/ml)	# of cells tested	MPC (μg/ml)
PA – 25	Skin and Soft tissue	2.1×10^6	≤0.125	2.1×10^6	0.5	Susceptible	5.0×10^{10}	1.75	5.0×10^{10}	9
RV – 85431	Sputum	5.8×10^5	0.25	5.8×10^5	1	Susceptible	4.4×10^{10}	3.25	4.4×10^{10}	12
RV – 77900	Abdominal fluid	8.0×10^5	≤0.125	8.0×10^5	0.5	Susceptible	4.8×10^{10}	2	4.8×10^{10}	8
Mt.S – 7	Urine	8.7×10^5	≤0.125	8.7×10^5	0.5	Susceptible	4.6×10^{10}	2.5	4.6×10^{10}	8
OGH – 4	Skin and Soft tissue	1.1×10^6	≤0.125	1.1×10^6	0.5	Susceptible	4.1×10^{10}	1.125	4.1×10^{10}	4
St.C – 14344	Sputum	3.2×10^5	0.5	3.2×10^5	1	Susceptible	2.1×10^{10}	2.5	2.1×10^{10}	11
Ave of 6 Susceptible Isolates		**9.6×10^5**	**0.21**	**9.6×10^5**	**0.7**	**Susceptible**	**4.2×10^{10}**	**2.2**	**4.2×10^{10}**	**8.7**
CBRH – 25138	Urine	1.0×10^6	2	1.0×10^6	4	Intermediate	5.7×10^{10}	8	5.7×10^{10}	16

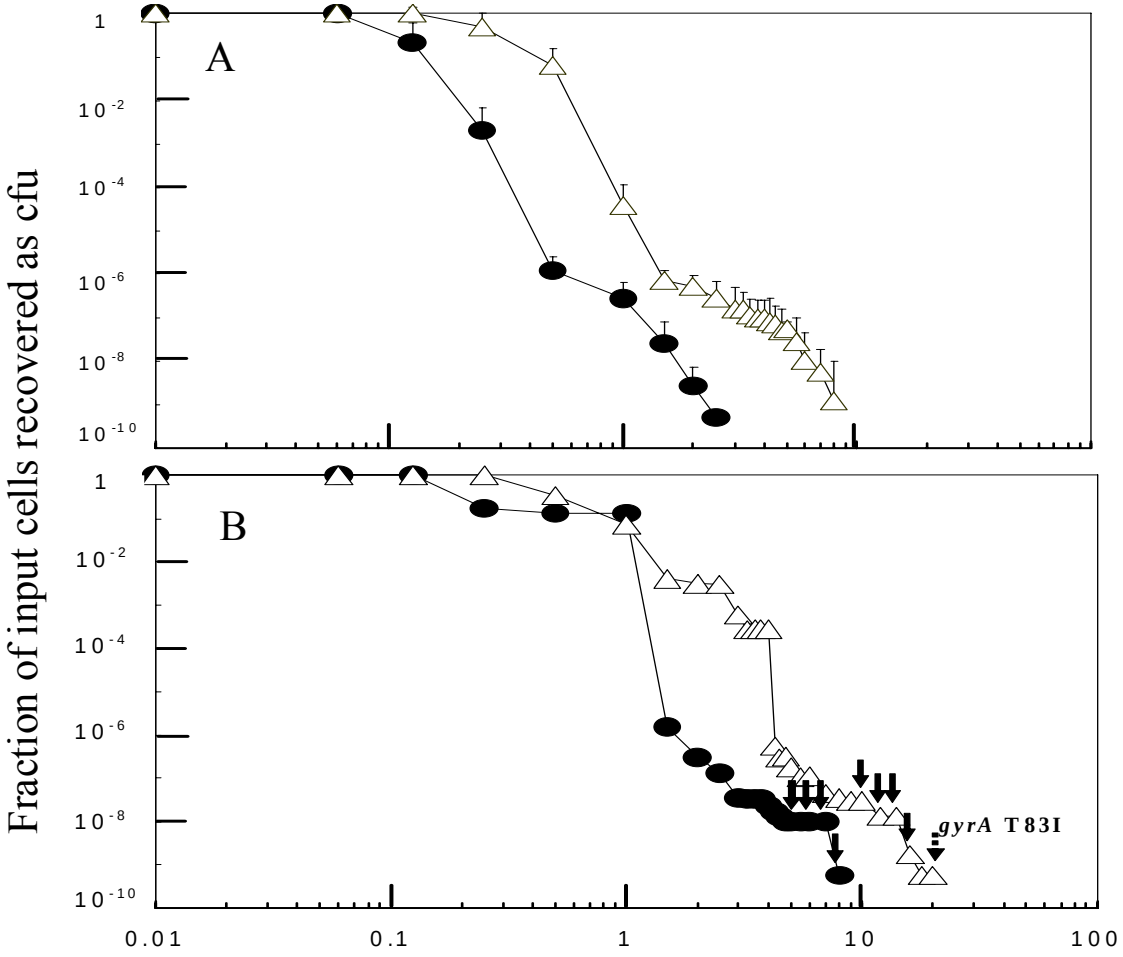
[a] MIC results obtained using microbroth dilution method. [b]Susceptibility based on NCCLS guidelines for susceptible (S), intermediate (I), resistant (R) (258). Ciprofloxacin S, I, R = ≤ 1, 2, ≥ 4 μg/ml. Levofloxacin S, I, R = ≤ 2, 4, ≥ 8 μg/ml.

activities for ciprofloxacin and levofloxacin could be quantitatively assessed by examining the numbers of mutants selected with respective ciprofloxacin and levofloxacin MSWs. Thus, the frequency with which mutants are selected by each drug could be quantitatively described in the relative area created during mutant selection. Levofloxacin was 1500 times (a > 3 log difference in CFU) more efficient at selecting mutant colonies of *P. aeruginosa* at comparable concentrations.

4.3.2 Effect of Fluoroquinolone Concentration on Mutant Recovery for a Clinical Isolate of *P. aeruginosa*

The effect of fluoroquinolone concentration of mutant recovery from a clinical isolate (CBRH 25138) with intermediate susceptibility to ciprofloxacin (MIC=2 μg/ml) and levofloxacin (MIC=4 μg/ml) was examined in reference to susceptible strains (Figure 4.3.2). As expected, the recovery of CFU that occurred for a strain with intermediate susceptibility resulted in a shift in the mutant selection curve toward higher concentrations (ciprofloxacin MPC= 8 μg/ml, levofloxacin MPC =16 μg/ml). At fluoroquinolone concentrations which approximated the MIC for ciprofloxacin (MIC= 2 μg/ml) and levofloxacin (MIC= 4 μg/ml), a sharp decline in colony recovery was seen which occurred for approximately half of the cells present in the inoculums tested (i.e., 10^5 CFU). In comparison with mutants recovered from susceptible strains, the plateau in mutant recovery for a strain with intermediate fluoroquinolone susceptible occurred over a broad concentration range for ciprofloxacin and levofloxacin, respectively. Mutant growth was observed at ciprofloxacin concentrations encompassing 2-to-7 μg/ml. Thus, the plateau in colony recovery seen with ciprofloxacin concentrations was shifted an average of 4-fold, in the direction of higher ciprofloxacin concentrations when compared to colony recovery in susceptible strains. For levofloxacin, mutant growth occurred over drug concentrations ranging from 3-to-15 μg/ml, which shifted the mutant selection curve by an average of 3-fold in the direction of higher levofloxacin concentrations. The MPC for ciprofloxacin and levofloxacin were

Figure 4.3.2 Effect of Fluoroquinolone Concentration on Mutant Recovery for Clinical Isolates of *P. aeruginosa*



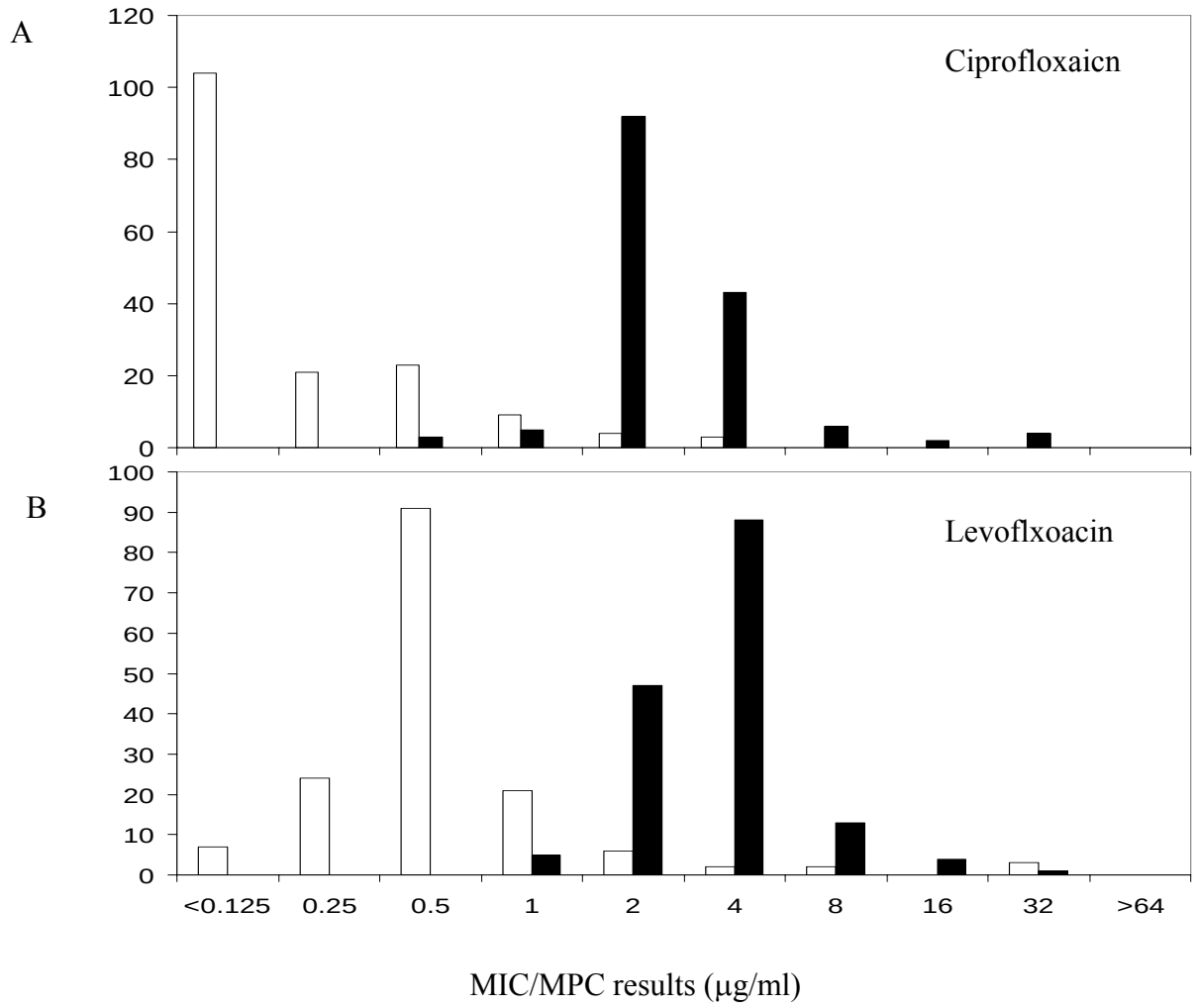
Clinical isolates were applied to tTSA plates containing the indicated concentrations of ciprofloxacin (filled circle) or levofloxacin (open triangle). The fraction of input cells recovered as mutants were determined after re-growth of colonies on drug-free agar followed by regrowth on agar containing the fluoroquinolone at the initial drug selection concentration. Each data point reflects the average decline in CFUs from six susceptible isolates of *P. aeruginosa* when 10^{10} CFU/ml inoculums when tested against a defined ciprofloxacin or levofloxacin concentration Panel A. represents the recovery of colonies *P. aeruginosa* colonies from six fluoroquinolone susceptible isolates of *P. aeruginosa*. Panel B. represents colony recovery from a 10^{10} CFU/ml culture of *P. aeruginosa* (isolate CBRH 25138) with intermediate susceptibility, ciprofloxacin MIC=2 μg/ml; levofloxacin MIC=4 μg/ml). Arrows indicate drug concentrations from which colonies were examined for *gyrA* and *parC* QRDR resistance mutations. A *gyrA* mutation was observed only at the point indicated.

measured at 3 and 8.5 μg/ml, respectively. Thus, the preferential ability of ciprofloxacin to restrict mutant growth was also evident with an isolate having intermediate susceptibility. Sequence analysis of 14 mutants (see arrows figure 4.3.2B) revealed that QRDR mutants (dashed arrow *gyrA* T83I) were selected at a levofloxacin concentration of 14 μg/ml (n=3 mutants). No QRDR mutants were recovered from selected mutants (n=6) recovered from agar plates containing ciprofloxacin concentrations.

4.3.3 Distribution of MIC and MPC Results for 155 Clinical Isolates of *P. aeruginosa*

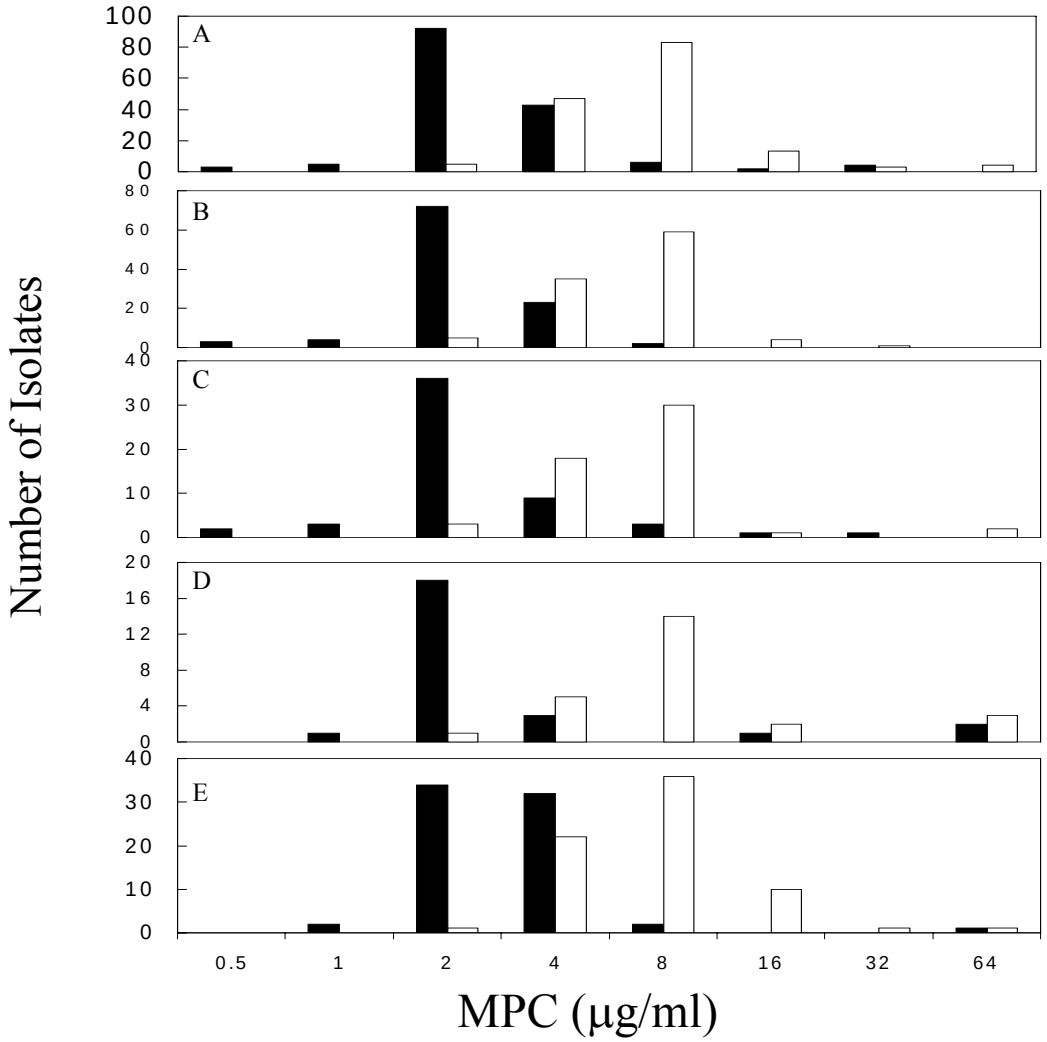
To obtain a more general view of fluoroquinolone susceptibility, I measured MIC and MPC values with 155 clinical isolates of *P. aeruginosa* using conventional 2-fold dilutional increments of ciprofloxacin and levofloxacin concentrations in agar plates (Figure 4.3.3). By traditional susceptibility measures the compounds were similar. MICs for ciprofloxacin ranged from 0.06-4 μg/ml, MIC_{50} and MIC_{90} values were ≤0.125 and 1μg/ml, respectively. Based on NCCLS breakpoints (258), 95%, 3% and 2% of the isolates, respectively, were susceptible, intermediate and resistant, to ciprofloxacin. MICs for levofloxacin ranged from 0.125-8 μg/ml, MIC_{50} and MIC_{90} values were 0.5 and μg/ml, with 96%, 1% and 3% considered susceptible, intermediate and resistant, respectively. MPC results for ciprofloxacin and levofloxacin were 4- to 32-fold higher than MIC results. As expected from data in Figure 4.3.1, the MPC for ciprofloxacin was lower than for levofloxacin (Figure 4.3.3; modal values were 2 and 8 μg/ml, respectively; values of MPC_{90} were 4 μg/ml and 16 μg/ml, respectively). Thus, the upper boundary of the mutant selection window (i.e., the MPC) was 4 times higher for levofloxacin. Ciprofloxacin was 2- to 8-fold more active than levofloxacin as determined by comparison of MPC results. The relative distributions of MPC results were affected only slightly by restricting the analysis to highly susceptible isolates (Figure 4.3.4) or in most cases by considering the anatomical site from which the isolates were obtained (Figures 4.3.4C-E). Urinary tract isolates displayed a shift in the

Figure 4.3.3 MIC and MPC Distributions for 155 Clinical Isolates of *P. aeruginosa* Against Ciprofloxacin and Levofloxacin



Panel A. depicts MIC (shaded bars) and MPC results (open bars) for 155 clinical isolates of *P. aeruginosa* against ciprofloxacin. Modal MIC and MPC values for ciprofloxacin were 0.125 and 0.5 μg/ml, respectively. Ciprofloxacin MIC_{90} and MPC_{90} values were 0.5 and 2 μg/ml, respectively. Panel B. depicts MIC (open bars) and MPC results (filled bars) for 155 clinical isolates of *P. aeruginosa* against levofloxacin. Modal MIC and MPC values were 0.5 and 8 μg/ml, respectively, and MIC_{90} and MPC_{90} were 1 and 16 μg/ml, respectively.

Figure 4.3.4 **MPC results for 155 Clinical Isolates of *P. aeruginosa* Against Ciprofloxacin and Levofloxacin**



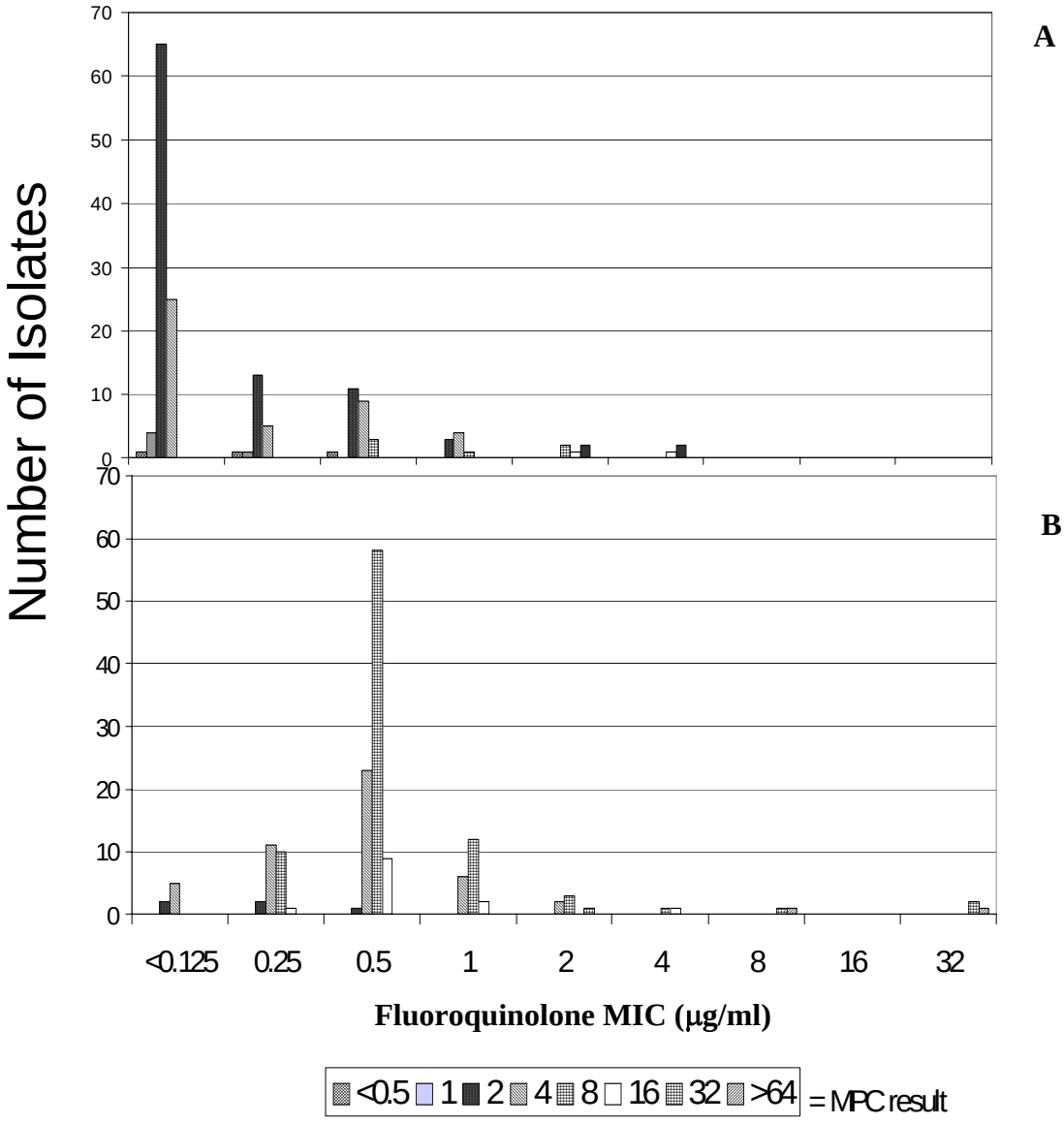
Distribution of MPC for clinical isolates of *P. aeruginosa*. Panel A. All isolates tested (n = 155). Panel B. Isolates having low values of MIC (≤ 0.5 μg/ml) to both ciprofloxacin and levofloxacin (n = 105). Panel C. Respiratory isolates (n = 55). Panel D. Skin and soft tissue isolates (n = 24), Panel E. Urinary tract isolates (n = 72). Four blood isolates are not shown. Similar results were obtained in a replicate experiment; solid bars indicate MPC for ciprofloxacin, open bars indicate levofloxacin. The modal MPC and MPC_{90} results for ciprofloxacin against respiratory, skin and soft tissue and urinary tract isolates of *P. aeruginosa* were 2 and 4 μg/ml, respectively; for levofloxacin these values were 8 and 16 μg/ml. The MPC distributions were largely unaffected by the source of isolation although a noticeable shift in the MPC distributions for urinary tract isolates was observed which is consistent with a history of ciprofloxacin use in UTIs.

distribution to higher drug concentrations; however, the shift in MPC distributions observed in the urinary subset of isolates did not change the overall MPC_{90} distribution.

4.3.4 Effect of Individual MIC Results on Ciprofloxacin and Levofloxacin MPC Distributions

Analysis of highly susceptible isolates of *P. aeruginosa* (MIC = ≤ 0.5 μg/ml) had little effect on the distribution of MPC results relative to the total population tested. I was interested in examining the effect of the initial degree of susceptibility (as assessed by the MIC) on the MPC distributions for ciprofloxacin and levofloxacin against clinical isolates of *P. aeruginosa.* Figure 4.3.5 shows the MPC distributions as they related to initial MIC results. As expected, isolates with low MIC tended to correlate with low MPC results. However, MIC results could not directly predict MPC results in some isolates and different MPC results were observed with isolates with identical MIC values. For example, 92/155 isolates of *P. aeruginosa* had a ciprofloxacin MPC of 2 μg/ml. Of the 92 isolates, 71% (65/92), 14% (13/92), 12% (11/92), and 3% (1/92), had ciprofloxacin MICs of ≤ 0.125, 0.25, 0.5, and 1, μg/ml, respectively.

Figure 4.3.5 Initial MIC Results and MPC Distributions for Ciprofloxacin and Levofloxacin for 155 Clinical Isolates of *P. aeruginosa*

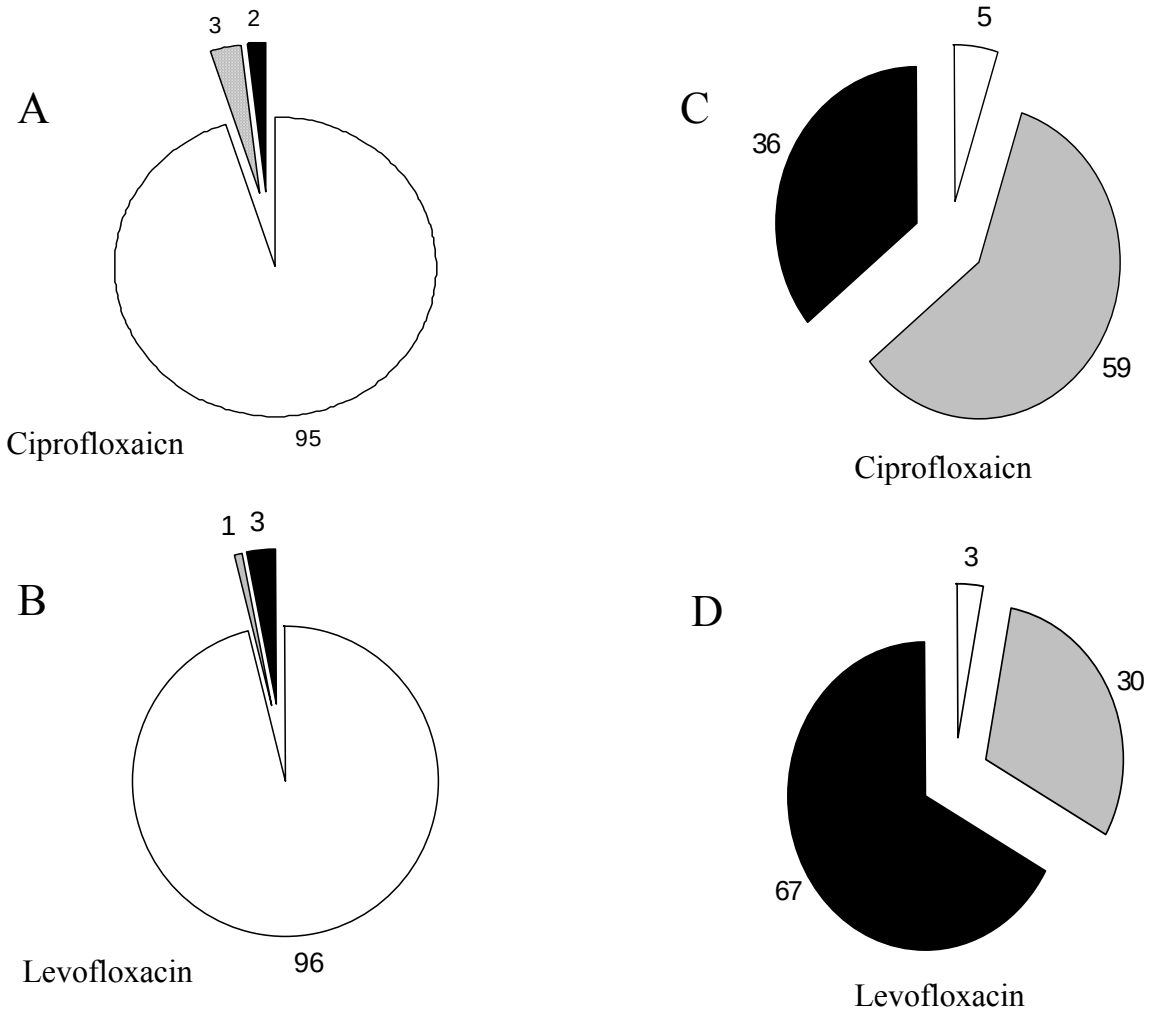


The number of isolates with corresponding MIC results to ciprofloxacin (panel A.) and levofloxacin (panel B.) are shown. The effect of MIC results on fluoroquinolone MPC distributions are depicted in shaded bars (see legend)

4.3.5 Relationship Between MPC Results and Susceptibility Breakpoints

MPC values can also be related to susceptibility breakpoints, which serve as empirical thresholds below which clinical success is expected to occur. NCCLS breakpoints for ciprofloxacin and levofloxacin were applied to MPC results for the 155 clinical isolates of *P. aeruginosa* (Figure 4.3.6). Breakpoints were determined based on NCCLS guidelines for ciprofloxacin and levofloxacin against *P. aeruginosa* (258). Briefly, results are reported as susceptible (S), intermediate (I), or resistant (R) (ciprofloxacin S, I, R = $\leq$ 1, 2, $\geq$ 4μg/ml and levofloxacin S, I, R = $\leq$ 2, 4, $\geq$ 8 μg/ml). Based on MIC results, both ciprofloxacin and levofloxacin had comparable susceptibility against clinical isolates of *P. aeruginosa.* For ciprofloxacin, the percentage of isolates conferring an S, I and R phenotype were 95%, 3% and 2%, respectively. For levofloxacin 96%, 1% and 3% of the isolates were classified as S, R, or I based on MIC results. Comparison of MPC results to NCCLS breakpoints for resistance revealed that 36% and 67% of the isolates tested against ciprofloxacin and levofloxacin would now be considered resistant. This finding suggests that neither antimicrobial agent would be appropriate for use as monotherapy in the treatment of *P. aeruginosa.* Based on MPC results, the percentage of isolates with an S, I, or R phenotype were 5%, 59% and 36% for ciprofloxacin and 3%, 30% and 67% for levofloxacin.

Figure 4.3.6 Relationship of MIC and MPC Results to NCCLS Susceptibility Breakpoints with *P. aeruginosa*



NCCLS breakpoints for resistance were applied to 155 clinical isolates of *P. aeruingosa* based on MIC and MPC results. Results are reported in percentage of total isolates. Susceptible (open areas), intermediate (shaded areas) or resistant (filled areas). Figures A and B illustrate MIC distributions for ciprofloxacin and levofloxacin, respectively. C and D illustrate MPC results for ciprofloxacin and levofloxacin, respectively.

4.3.6 Characterization of First-Step Mutants

4.3.6.1 Phenotypic Demonstration of Resistance in Recovered Mutants

I screened 204 mutants recovered from ciprofloxacin and levofloxacin containing plates using standardized microbroth dilution methods in order to examine the stability and susceptibility of recovered mutant colonies. The MICs of selected mutants were recorded as "$MIC_{recovered}$ " values and compared to the MIC of the parental strains (Table 4.3.2). For ciprofloxacin, the modal MIC of the original susceptible population was $\leq$ 0.125 μg/ml, and the MIC_{90} was 0.5 μg/ml. Of the original parental population, 90% (139/155) were inhibited by ciprofloxacin concentrations of $\leq$ 0.5 μg/ml. The modal $MIC_{recovered}$ and $MIC_{90\ recovered}$ results for ciprofloxacin were 2 and 4 μg/ml, respectively, which represented an 8-fold decrease in ciprofloxacin susceptibility relative to parental isolates. Of the recovered mutant population, only 3% (5/204) were inhibited by ciprofloxacin concentrations $\leq$ 0.5 μg/ml, compared to the MIC for 90% of the parental isolates. The levofloxacin modal MIC and MIC_{90} values for parental isolates of *P. aeruginosa* were 0.5 and 1 μg/ml, respectively, and 92% (142/155) of the parental isolates were inhibited by levofloxacin concentrations of $\leq$ 1 μg/ml. In comparison, the modal levofloxacin $MIC_{recovered}$ and $MIC_{90\ recovered}$ values for recovered mutant colonies were 4 and 16 μg/ml, and 3% (5/155) of the recovered mutants were inhibited by a levofloxacin concentration of $\leq$ 1 μg/ml. $MIC_{90\ recovered}$ results for mutant colonies possessed stable phenotypes, which were in 90% agreement with the MPC of the parental isolates. Overall, 81% (165/204) of the recovered mutants demonstrated a

Table 4.3.2 MIC and $MIC_{recovered}$ Results for Selected Mutants and Parental Strains of *P. aeruginosa*

FQ	Susceptibility Result (μg/ml)	Fluoroquinolone Susceptibility (μg/ml) summarized by MIC or MPC distributions										MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	MPC_{50} (μg/ml)	MPC_{90} (μg/ml)
Cipro		≤0.125	0.25	0.5	1	2	4	8	16	32	≥64				
	MIC (parental strains) (n=155)	95 (61%)	20 (74%)	23 (90%)	9 (95%)	4 (98%)	3 (100%)					≤0.125	0.5	---	---
	MIC (Recovered) (n=204)		1 (0.5%)	5 (3%)	18 (12%)	89 (55%)	72 (91%)	6 (94%)	5 (96%)	5 (99%)	3 (100%	2	4	----	----
	MIC –CCCP (n=65)		2 (3%)		4 (9%)	25 (23%)	20 (75%)	13 (95%)	1 (97%)	1 (99%)	1 (100%	4	8	----	----
	MIC +CCCP (n=65)		2 (3%)		7 (14%)	30 (60%)	16 (85%)	8 (97%)	1 (99%)	0	1 (100%	2	8	----	----
	MPC (n=155)			3 (3%)	5 (5%)	92 (65%)	43 (92%)	6 (96%)	2 (97%)	4 (100%				2	4
Levo	MIC (parental strain) (n=155)	7 (5%)	24 (20%)	91 (79%)	20 (92%)	6 (96%)	2 (97%)	2 (99%)		3 (100%		0.5	1	8	16
	MIC (Recovered) (n=204)		1 (0.5%)		4 (3%)	9 (7%)	63 (38%)	106 (90%)	6 (93%)	9 (97%)	6 (100%	8	16	----	----
	MIC –CCCP (n= 64)				1 (0.5%)	3 (6%)	4 (13%)	33 (64%)	20 (95%)	2 (99%)	1 (100%	8	16	----	----
	MIC +CCCP (n= 64)				1 (0.5%)	3 (6%)	10 (22%)	30 (69%)	19 (98%)	0	1 (100%	8	16	----	----
	MPC (n=155)					5 (3%)	47 (34%)	84 (88%)	13 (96%)	4 (97%)	1 (100%			8	16

FQ= fluoroquinolone; Cipro = ciprofloxacin, Levo = levofloxacin. MIC results for the paretnal populations and recovered mutants were analyzed. Recovered mutants demonstrated elevated MIC results with respect to the parental organisms when re-tested by conventional microbroth dilution. $MIC_{recovered}$ results were in 90% agreement with previously measeured MPC results. A 50 mM amount of CCCP was added to the wells of microtitre tray containing increasing concentrations of ciprofloxacin and levofloxacin. The distributions of MIC results are shown.

$\geq$ 2-fold change in the MIC to either ciprofloxacin or levofloxacin when tested by the conventional microbroth dilution.

The addition of a 50 mM solution of CCCP, to the wells of the susceptibility panels has been documented as a method for implicating possible efflux mechanisms which contribute to fluoroquinolone resistance by inhibting proton motive force (287, 365). Generally, a > 2-fold change in the MIC upon the addition of CCCP to the wells of the microtitre tray is required to implicate the possible involvement of efflux as contributing of fluoroquinolone resistance (365). By this system, active efflux dose not appear to be a major mechanism of fluoroquinolone resistance in recovered mutants (Table 4.3.2). For ciprofloxacin, 9% (6/65) of the recovered mutants demonstrated a > 2-fold change in the MIC when CCCP was added. For levofloxacin, 11% (7/64) of the recovered mutant population demonstrated a > 2-fold change in the MIC when CCCP was added to wells containing levofloxacin drug concentrations. Recovered mutants were tested for altered susceptibility patterns relative to parental organism by a semi-automated laboratory identification system know as Vitek. No differences between the ciprofloxacin and levofloxacin susceptibilities for parental organisms and recovered colonies were seen (data not shown).

4.3.6.2 DNA Sequence Analysis of *gyrA* and *parC* Targets in Recovered Mutants of *P. aeruginosa*

Some of the isolates required high fluoroquinolone concentrations ($\geq$ 8 μg/ml) to prevent colony formation. Analysis of the MIC results for the recovered mutants confirmed elevated susceptibility patterns with respect to parental organism in the presence of fluoroquinolones and CCCP. Thus, I suspected that these some of these organisms contained mutations in the target genes, *gyrA* (DNA gyrase) and/or *parC* (topoisomerase IV). To test this idea, I sequenced a 484 base-pair region of the gyrA gene and a 424 base-pair region of the parC which included the QRDR of each gene (see Materials and Methods). Twenty-five different mutants, from 11 different isolates were analyzed along with the original parental colony and mutations were reported in reference to target sequences in the parental isolate (Table 4.3.3). Low concentrations of fluoroquinolones selected only non-GyrA mutants; at slightly higher concentrations, distinct *gyrA* and *parC* variants were obtained. A number of mutations which have not been associated with quinolone resistance (non-characterized mutations) were identified in mutant colonies. To my knowledge, genetic studies have not been preformed that attribute resistance to these alleles, but they represent mutations which are not seen in parental organisms, consequently, I did not exclude these mutations from my analysis. Five mutants (isolate numbers 13, 27, 77, 105, and 126) contained a *gyrA* mutation (T83I) known from genetic studies to confer resistance (4). A number of uncharacterized silent mutations were identified which were not present in the sequences

Table 4.3.3 Sequence Analysis of GyraseA and TopoisomeraseIV Target Genes of Recovered Mutants of *P. aeruginosa*

Isolate (source)	Wild type MIC (μg/ml)		MPC (μg/ml)		QRDR Amino Acid Sequence	
	Cipro	Levo	Cipro	Levo	*gyrA*	*parC*
13 (LEVO-8μg/ml)	0.5	2	2	16	**T83I ACC to ATC)** H132H (CAC to CAT)	N27N (AAT to AAC) A115A (GCT to GCG)
27 (LEVO-4μg/ml)	1	2	8	16	Wild type	Wild type
27 (LEVO-5μg/ml)	1	2	8	16	Wild type	Wild type
27 (LEVO-7μg/ml)	1	2	8	16	Wild type	Wild type
27 (LEVO-10-a μg/ml)	1	2	8	16	Wild type	Wild type
27 (LEVO-10-b μg/ml)	1	2	8	16	**T83I ACC to ATC)** A84A (GCG to GCA)	Wild type
27 (LEVO-12 μg/ml)	1	2	8	16	**T83I ACC to ATC)** A84A (GCG to GCA)	Wild type
27 (LEVO-16 μg/ml)	1	2	8	16	**T83I ACC to ATC)** A84A (GCG to GCA)	Wild type
27 (CIPRO -2 μg/ml)	1	2	8	16	Wild type	Wild type
27 (CIPRO – 3.μg/ml)	1	2	8	16	Wild type	Wild type
27 (CIPRO –3.5 μg/ml)	1	2	8	16	Wild type	Wild type
40 (LEVO-16μg/ml	≤0.125	1	8	32	H132H (CAC to CAT)	N27N (AAT to AAC)

						A115A (GCT to GCG)
40 (CIPRO 4μg/ml	≤0.125	1	8	32	H132H (CAC to CAT)	N27N (AAT to AAC) A115A (GCT to GCG)
76 (LEVO 4 μg/ml)	0.5	1	2	8	A67A (GCC to GCG) H132H (CAC to CAT)	A115A (GCT to GCG)
76 (CIPRO 1 μg/ml)	0.5	1	2	8	Wild type	Wild type
77a (CIPRO-32μg/ml)	1	2	>32	>32	**T83I ACC to ATC)**	A115A (GCT to GCG)
*#77a (LEVO-32μg/	1	2	>32	>32	**T83I ACC to ATC)** H132H (CAC to CAT)	N27N (AAT to AAC) A115A (GCT to GCG)
81 (LEVO-16μg/ml)	≤0.125	0.5	8	32	H132H (CAC to CAT)	
85 (LEVO-16μg/ml)	≤0.125	0.125	8	32	H132H (CAC to CAT)	N27N (AAT to AAC) A115A (GCT to GCG)
#92 (LEVO-8μg/ml)	≤0.125	1	4	16	G105G (GGC to GGT); H132H (CAC to CAT)	N27N (AAT to AAC) A115A (GCT to GCG)
#105 (LEVO-32μg/ml)	1	2	>32	>32	**T83I ACC to ATC**	N27N (AAT to AAC) A115A (GCT to GCG)
#103 (LEVO-8μg/ml	0.25	1	4	16	V103V (GTA to GTC) A118A (GCA to GCG) A136A (GCG to GCC) D144D (GAC to GAT) D150D (GAT to GAC)	N27N (AAT to AAC); H40H (CAT to CAC) R50R (CGA to CGG) D62D (GAT to GAC) Y107Y (TAT to TAC) G114G (GGG to GGC) Y135Y (TAT to TAC)
103 (CIPRO - 1μg/ml)	0.25	1	2	16	Wild type	Wild type
126 (LEVO-	1	2	2	16	**T83I ACC to ATC**;	N27N (AAT to AAC)

8μg/ml)						A115A (GCT to GCG)
126 (CIPRO-1μg/ml)	1	2	2	16	H132H (CAC to CAT)	N27N (AAT to AAC) A115A (GCT to GCG)

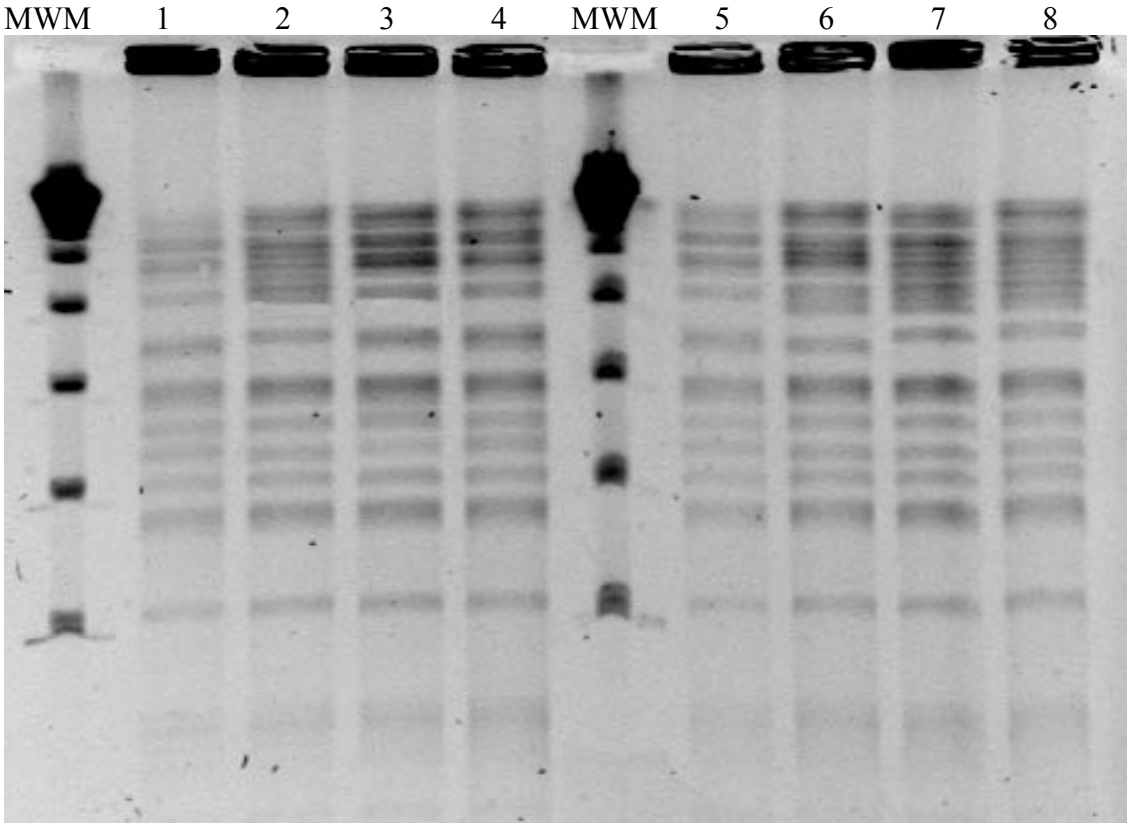
Nucleotide sequences were determined for the QRDRs of *P. aeruginosa gyrA* and *parC* genes (3, 195) using PCR and automated DNA sequencing. [a] Cipro = ciprofloxacin; Levo =levofloxacin. [b] QRDR, quinolone-resistance-determining region. Amino acid in wild-type protein is indicated before its number in the protein, followed by the amino acid change. A, alanine; D, aspartic acid; H, histadine; G, glycine; I, Isoleucine; N, asparagine; R, arginine; T, threonine Y, tyrosine; V, valine. All mutations are reported in reference to changes from the orginal parental sequence.

of the parental organisms. For example, a *gyrA* H132H (CAC-CAT) and a *parC* mutation N27N (AAT- AAC) where identified in mutant colonies. The type of *gyrA* and *parC* mutation selected also differed for mutants recovered from ciprofloxacin and levofloxacin plates. For isolate 126, a T83I *gyrA* mutation was identified in a mutant recovered from an agar plate containing 8 μg/ml of levofloxacin. This mutant was not recovered from colonies selected from any plates containing ciprofloxacin.

4.3.6.3 PFGE Analysis of Recovered Mutants of P. *aeruginosa*

MIC testing confirmed the presence of stable phenotypes in mutant colonies and DNA sequence analysis of the QRDR for the *gyrA* and *parC* genes revealed target mutations. I used PFGE to test the hypothesis that mutant colonies represented different clones of *P. aeruginosa* compared to parental isolates. PFGE DNA banding patterns for 4 mutants recovered from agar plates containing levofloxacin concentrations were examined and compared to the PFGE fingerprint of the parental isolates. For all mutants tested, no differences in the PFGE pattern were observed between mutant and parental colonies selected from 10^{10} CFU/ml cultures of *P. aeruginosa* (Figure 4.3.7).

Figure 4.3.7 PFGE Analysis of Recovered Mutants of *P. aeruginosa*



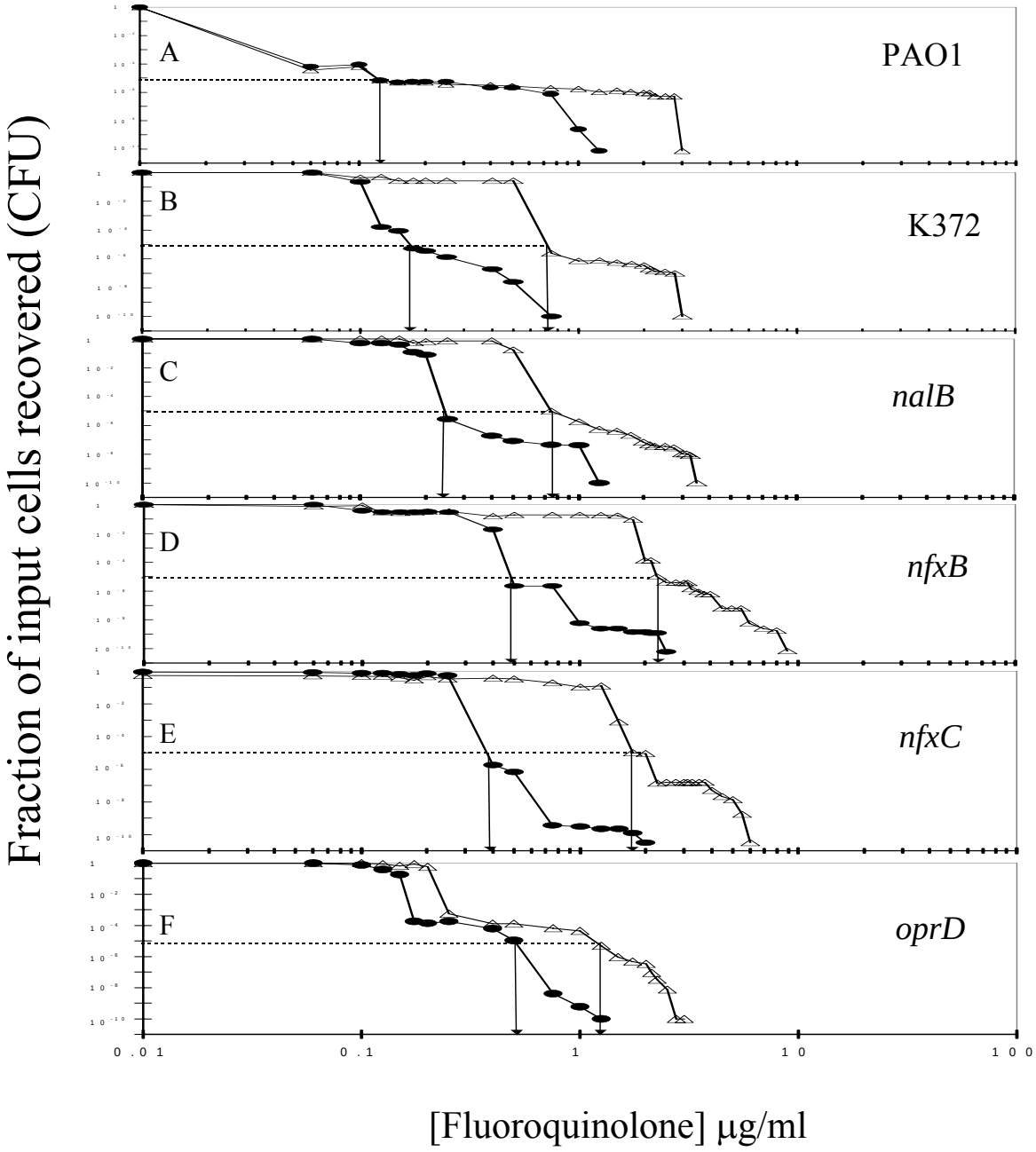
Lane #	Recovered mutant	MIC (μg/ml)		MPC (μg/ml)	
		Cpx	Lfx	Cpx	Lfx
(MWM) = 123 base pair molecular weight marker					
1 = PA- CMH 32 (parental strain)	levo-4 μg/ml	1	2	4	8
2 = PA- CMH 32 (recovered mutant)					
3 = PA-OGH 22 (parental strain)	levo-16 μg/ml	4	8	32	≥32
4= PA- OGH 22 (recovered mutant)					
5= PA- HMR 24 (parental strain)	levo-4 μg/ml	1	4	2	8
6= PA- HMR 22 (recovered mutant)					
7= PA –OGH 17 (parental strain)	levo-16 μg/ml	2	4	8	32
8= PA- OGH 17 (recovered mutant)					

PFGE was preformed on four clinical strains of *P. aeruginosa.* Cpx = ciprofloxacin. Lfx = levofloxacin.

4.3.7 Enrichment of Mutant Subpopulations from *nalB, nfxB, nfxC,* and *oprD* Mutants of *P. aeruginosa*

Some of the recovered mutants demonstrated elevated susceptibility in the absence of alterations in sequenced regions of the *gyrA* and *parC* genes. Phenotypic analysis of mutants recoved at low and moderate fluoroquinolone concentrations using mutant selection curves suggest that the nature of the low-concentration mutant slection may be efflux mediated. Why the non-gyrase mutants were so abundant has not been established. I examined the possibility that differences in mutant selection between ciprofloxacin and levofloxacin may be partially attributed to the ability to inhibit cells containing efflux mechanisms. Mutant selection curves were performed on *nalB, nfxB, nfxC,* and *oprD* efflux mutants of *P. aeruginosa* (Figure 4.3.8). For each strain tested, a two-stage decline in growth was observed. The first stage in colony decline occurred at fluoroquinolone concentrations that approximated the ciprofloxacin and levofloxacin MIC, and resulted in the inhibition of growth of approximately 10^5 cells (see arrows, Figure 4.3.8). At concentrations above the ciprofloxacin and levofloxacin MIC of the strain tested, growth of mutants occurred. Eventually, a concentration which inhibited the most-resistant mutant present in 10^{10} CFU/ml populations was obtained, defining the MPC for the fluoroquinolones tested. For the *nalB* mutant (MexAB-OprM), the MPC for ciprofloxacin and levofloxacin was 1.125 and 3.5 μg/ml,

Figure 4.3.8 Effect of Fluoroquinolone Drug Concentration on Colony Recovery from n*alB, nfxB ,nfxC,* and o*prD* efflux Mutants of *P. aeruginosa*



P. aeruginosa cultures containing 10^{10} CFU/ml were applied to agar plates containing increasing concentrations of fluoroquinolones. PAO1 isogenic strain for the *nalB, nfxB* and *oprD* efflux mutants. K372 isogenic strain for the *nfxC* mutant. *NalB* (MexAB-OprM) efflux mutant. *NfxB* (MexCD-OprN) efflux mutant. *NfxC* (MexEF-OprJ) efflux mutant. *OprD* efflux mutant. Ciprofloxacin is represented by filled circles; levofloxacin is represented by open triangles. Arrows represent the MIC for each strain tested.

respectively, which represents a 2- to 3-fold decrease in susceptibility relative to the MIC of the wild type strain (ciprofloxacin MIC= 0.25 μg/ml, levofloxacin MIC = 0.75 μg/ml) (Table 4.3.4). Based on the MPC result, ciprofloxacin was 3.1-fold more active against mutants selected from 10^{10} CFU/ml cultures of a *nalB* mutant of *P. aeruginosa*. Differences between the numbers of mutants selected by ciprofloxacin and levofloxacin, above the MIC of the wild-type strain, were not remarkable. However, the concentrations of levofloxacin required to inhibit mutant colonies were 2-to 4.5-fold higher than for ciprofloxacin, resulting in a higher levofloxacin MPC, and a lower AUC/MPC ratio (levofloxacin AUC/MPC = 23, ciprofloxacin AUC/MPC = 35)

The highest fluoroquinolone MPCs were measured against the *nfxB* mutant (MexCD-OprJ) which corresponded to ciprofloxacin and levofloxacin MPCs of 2.5 and 9 μg/ml, respectively, and represented a 5- to 4-fold decrease in susceptibility to ciprofloxacin and levofloxacin relative to the MIC of the parental strain (ciprofloxacin MIC = 0.5 μg/ml, levofloxacin MIC = 2.25 μg/ml) (Table 4.3.4). AUC/MPC values of ciprofloxacin and levofloxacin for an *nfxB* mutant were the lowest among all the efflux mutants tested and corresponded to a ciprofloxacin value of 18 and levofloxacin value of 9. Analysis of mutant selection, based on the MSW, for ciprofloxacin and levofloxacin revealed that levofloxacin selected 12.6 times the number of mutant

Table 4.3.4 MIC and MPC Results for n*alB, nfxB, nfxC* and o*prD* Efflux Mutants of *P. aeruginosa*

Strain	**Fluoroquinolone**	**MIC (μg/ml)**	**MPC (μg/ml)**	**Change in Susceptibility (fold increase)**	*AUC/MPC*
nalB	Ciprofloxacin	0.25	1.125	4.5	35
	Levofloxacin	0.75	3.5	4.6	23
nfxB	Ciprofloxacin	0.5	2.5	5	18
	Levofloxacin	2.25	9	4	9
nfxC	Ciprofloxacin	0.4	2	5	22
	Levofloxacin	1.75	6	3.4	14
oprD	Ciprofloxacin	0.5	1.125	2.25	35
	Levofloxacin	1.25	2.5	2	47
PAO1[a]	Ciprofloxacin	0.125	0.75	6	59
	Levofloxacin	0.75	3.125	4.2	26
K372[b]	Ciprofloxacin	0.175	1	5.7	44
	Levofloxacin	0.75	3	4	27

[a]PAO1 is the isogenic strain for the *nalB, nfxB,* and *oprD* mutants
[b]K372 is the isogenic strain for the *nfxC* mutant

colonies when compared to selction of mutants with ciprofloxacin drug concentrations. Fluoroquinolone concentrations at which mutant selection occurred were 3.6- to 5-fold higher for levofloxacin. Thus, levofloxacin continued to select mutants over a resistant breakpoint for the drug. In contrast, mutants selected by ciprofloxacin concentrations were below the breakpoint for resistance. Comparisons between the *nfxB* mutant and the isogenic strain (PAO1), revealed no differences in the frequency of mutant selection. Comparison of MPC results revealed a 3-fold increase between the *nfxB* mutant and the respective isogenic strain. Ciprofloxacin and levofloxacin MPC results for the *nfxC* mutant (MexEF-OprN), were 2 and 6 μg/ml, respectively. MexEF-OprN efflux expression caused a 5- and 3.4-fold decrease in the susceptibility to ciprofloxacin and levofloxacin. A fourth efflux mutant, o*prD*, which confers resistance to carbapenem antibiotics via reductions in porin channels, resulted in small changes to fluoroquinolone susceptibility when MIC and MPC results were compared (2.2-fold difference for ciprofloxacin, 1.8-fold difference for levofloxacin). The MPC results for ciprofloxacin and levofloxacin were 1.25 and 3.125 μg/ml, respectively (Table 4.3.4).

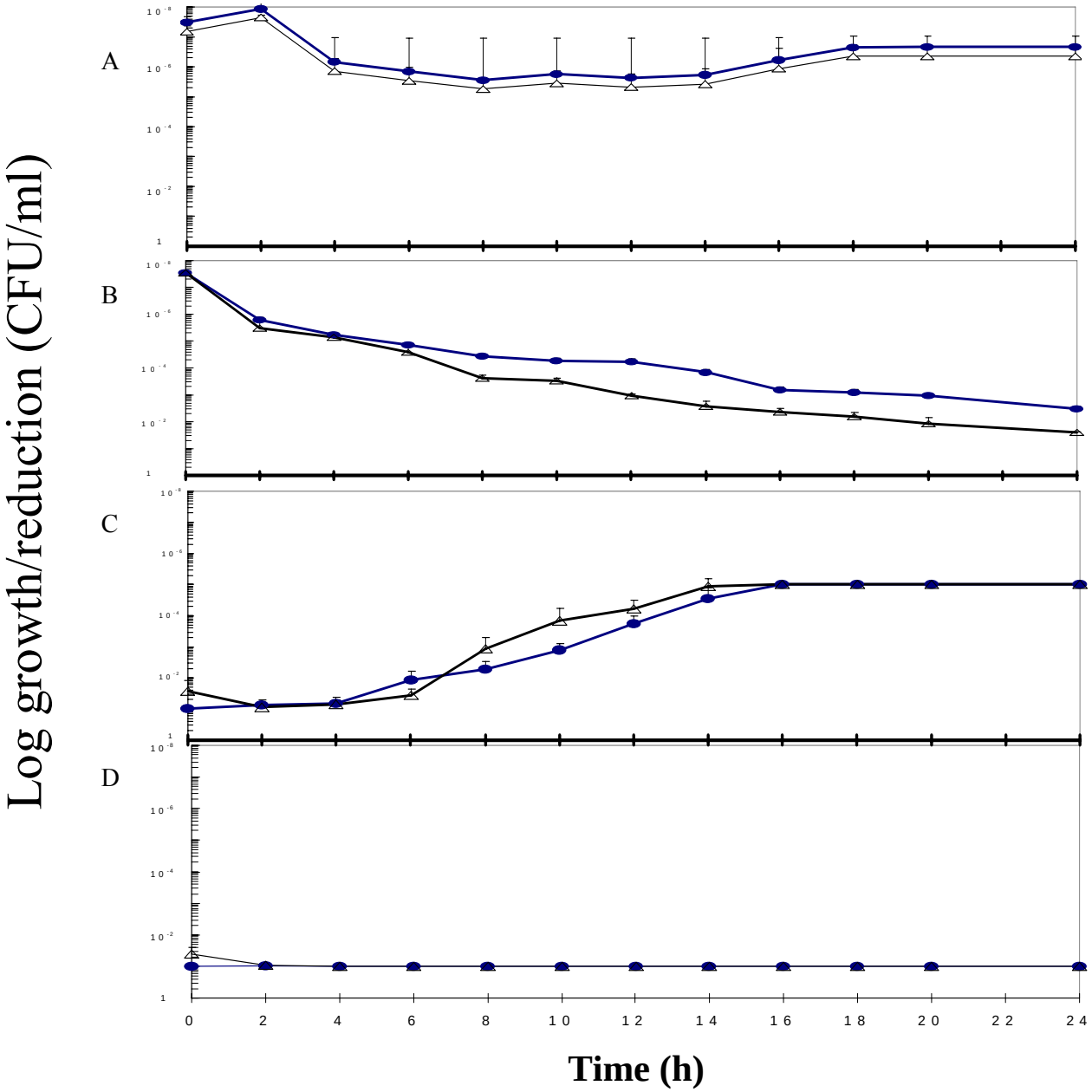
Selection of first-step resistant mutants from isolates of *P. aeruginosa* expressing efflux mechanisms resulted in higher ciprofloxacin and levofloxacin MPC results when compared to the MPC of the respective isogenic strains. Two mutants which overexpressed the MexCD-OprJ (*nfxB*) and MexEF-OprN (*nfxC*) operons resulted in high MPC values which were above the breakpoints for resistance for

ciprofloxacin and levofloxacin, respectively. Sequence analysis for regions of the *gyrA* and *parC* genes in selected mutants have not been performed, but selection of resistant mutants which co-express efflux plus target mutations are likely to have high MPCs. Ciprofloxaicn had lower MPCs than levofloxacin against strains of *P. aeruginosa* which express drug efflux systems, suggesting that the ability to restrict the selection of fluoroquinolone-resistant mutants may be linked to the ability to inhibit efflux expression in clinical isolates of *P. aeruginosa.*

4.3.8 Bacterial Killing and MPCs for Clinical Isolates of *P. aeruginosa*

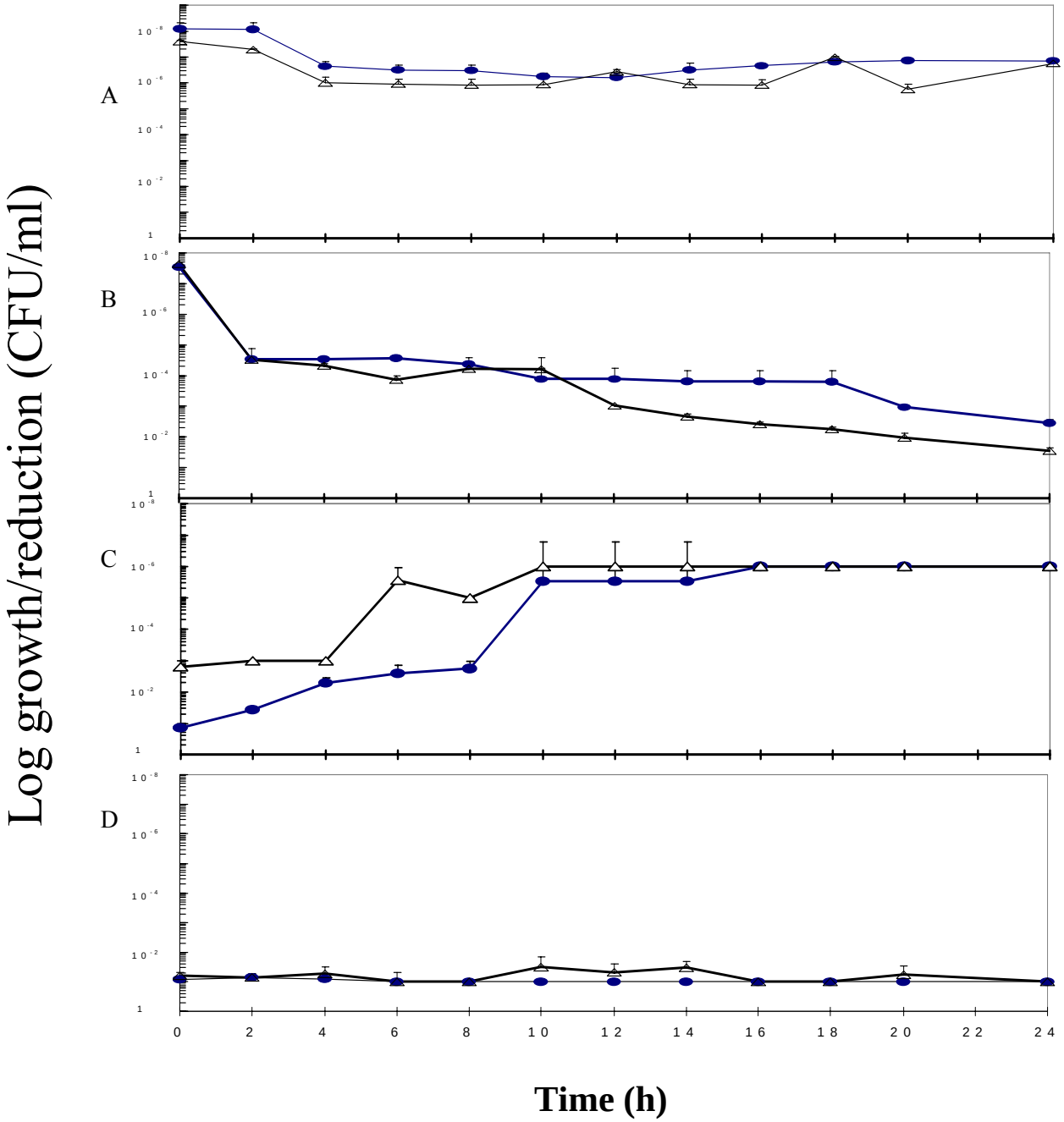
Like other susceptibility measurements, the MPC is not reflective of bacterial killing and the time that fluoroquinolones are required to exceed the MPC during dosing is a factor which is currently unknown. It is expected that first-step mutants selected at sub-MPC drug concentrations may be selectively amplified under dosing which allows drug concentrations to fluctuate within the MSW. I examined the effect of the MPC drug concentration on the killing of 10^{10} CFU/ml cultures of *P. aeruginosa* and the killing/enrichment of mutant populations. Killing experiments for *P. aeruginosa* were preformed as outline in Materials and Methods. Briefly, the contents of 10^{10} CFU/ml cultures were evenly divided among two 500 ml flasks containing the specified MPC or MIC drug concentrations. Mutant growth was assessed via growth on agar plates containing concentrations of ciprofloxacin and levofloxacin that were an average of 4-fold above the MICs of the wild-type strains. Experiments were preformed in duplicate and conducted over 24 hr. The rates of killing at the MIC and MPC drug concentrations for two strains of *P. aeruginosa* (CBRH- 25138 and PA-25) are shown in Figures 4.3.9 and 4.3.10. For each experiment the detection limit was +1.00 log growth. The MPC for ciprofloxacin and levofloxacin against *P. aeruginosa* isolates CBRH 25138 and PA-25 were 4 and 16, and 2 and 4 μg/ml, respectively. Figure 4.3.9 and Table 4.3.5 depict results obtained for the *P. aeruginosa* strain CBRH-25138. At the MIC, -1.32 and -1.33

Figure 4.3.9 **Killing of *P. aeruginosa* CBRH 25138 by Ciprofloxacin and Levofloxacin at the MIC and MPC**



The killing of *P. aeruginosa* CBRH-25138 at the MIC and MPC are represented in panels A and B. The killing of mutants at the MIC and MPC drug concentrations are represented in panels C and D. Open triangles represent the growth/reduction in viablility for levofloxacin-treated cultures. Filled circles represent the growth/reduction in viablility for ciprofloxacn-treated cultures.

Figure 4.3.10 Killing of *P. aeruginosa* PA-25 by Ciprofloxacin and Levofloxacin at the MIC and MPC



The killing of *P. aeruginosa* PA-25 at the MIC and MPC are represented in panels A and B. The killing of mutants at the MIC and MPC are represented in panels C and D. Open triangles represent the growth/reduction in viablility for levofloxacin-treated cultures. Filled circles represent the growth/reduction in viablility for ciprofloxacin-treated cultures.

Table 4.3.5 Killing of *P. aeruginosa* CBRH 25138 by Ciprofloxacin and Levofloxacin at the MIC and MPC

Original Strain	Killing at the MIC (μg/ml)	Fluoroquinolone	Time (h)	Original broth concentration (CFU/ml)	Log Reduction/growth of entire culture (CFU/ml)	Log Reduction/ growth of mutant growth (CFU/ml)
CBRH 25138	MPC_{cipro} =2 MPC_{levo} =4	Ciprofloxacin Levofloxacin	0	3.06×10^7 1.53×10^7	------- [a] ---------- b	+1.01 [c] +1.58 [d]
		Ciprofloxacin Levofloxacin	4	1.45×10^6 7.23×10^5	-1.32 -1.33	+1.18 +1.15
		Ciprofloxacin Levofloxacin	8	3.56×10^5 1.78×10^5	-1.93 -1.93	+2.29 +2.93
		Ciprofloxacin Levofloxacin	12	4.2×10^5 2.1×10^5	-1.86 -1.87	+3.75 +4.22
		Ciprofloxacin Levofloxacin	18	4.44×10^5 2.22×10^6	-1.84 -1.83	+≥6.00 +≥6.00
		Ciprofloxacin Levofloxacin	24	4.58×10^6 2.29×10^6	-0.82 -0.83	+≥6.00 +≥6.00
Original Strain	**Killing at the MPC (μg/ml)**	**Fluoroquinolone**	**Time (h)**	**Original broth concentration (CFU/ml)**	**Log Reduction/growth of entire culture (CFU/ml)**	**Log Reduction/ growth of mutant growth (CFU/ml)**
CBRH 25138	MPC_{cipro} =8 MPC_{levo} =16	Ciprofloxacin Levofloxacin	0	3.41×10^7 3.41×10^7	------- [e] ---------- f	+1.00 [c] +1.40 [d]
		Ciprofloxacin Levofloxacin	4	1.76×10^5 1.37×10^5	-2.28 -2.40	+1.00 +1.00
		Ciprofloxacin Levofloxacin	8	2.71×10^4 4.12×10^3	-3.10 -3.92	+1.00 +1.00
		Ciprofloxacin Levofloxacin	12	1.68×10^4 9.25×10^2	-3.31 -4.57	+1.00 +1.00
		Ciprofloxacin Levofloxacin	18	1.20×10^3 1.58×10^2	-4.45 -5.33	+1.00 +1.00
		Ciprofloxacin Levofloxacin	24	3.02×10^2 4.00×10^1	-5.05 -5.93	+1.00 +1.00

Maximum log reduction; [a]-7.49, [b]- 7.18. [e]-7.53, [f]-7.53.
Maximum log growth; [c]+ ≥6.0, [d]+ ≥6.0.

log reductions in growth were measured for the strain CBRH 25138 for ciprofloxacin and levofloxacin by 4 hr. Maximum killing for both ciprofloxacin and levofloxacin occurred by 8 hr. Neither ciprofloxacin nor levofloxacin treated cultures achieved >3 log reductions in viable cells at any time tested at the MIC drug concentration. By 24 hr, a -0.82 and -0.83 log reduction in viable counts were observed for ciprofloxacn and levofloxacin, respectively. As expected, an increase in killing occurred when the MPC drug concentration was tested. By 4 hr, a -2.28 and -2.4 log reduction in growth was observed for ciprofloxacin and levofloxacin. Significant killing, as assessed by $\geq$ -3.0-log reduction, was measured at 8 hr and corresponded to -3.10 and -3.90 log reductions in growth for ciprofloxacin and levofloxacin, respectively. Maximal killing was observed by 24 hr which resulted in -5.05 and -5.93 log reduction in viable cells when ciprofloxacn and levofloxacin were tested.

For the *P. aeruginosa* strain CBRH-25138, mutant growth was determined via conoly formation on agar plates containing 4 and 8 μg/ml of ciprofloxaicn and levofloxacin, respectively. At time 0 hr, mutatnt colonies were detected in three of four cultures tested and demonstrated that mutant growth was present prior to introduction of a fluoroquinolone. Mutant colonies were recovered from both ciprofloxaicn and levofloxacin-treated cultures at every time interval tested. By 8 hr, a 2.29 and 2.93 log increase in mutant growth was observed, and by 18 hr, mutant growth reached the detection limit ($\geq$ +6.00 log) of the assay. At the MPC drug concentration, mutant

growth was initially detected at time 0 hr in 3 of 4 culutres. However, no mutants were recovered at time points beyond 2 hr for either ciprofoxaicn or levofloxacin treated cultures (Figure 4.3.9).

For the *P. aeruginosa* strain PA-25 (Figure 4.3.10), killing by 24 hr at the MIC drug concentration for ciprofloxacin and levofloxacin was -1.25 and -1.83 log, respectively (Table 4.3.6). A $\geq$ -3 log reduction in growth for ciprofloxaicn or levofloxacin was not obtained at any point sampled during 24 hr and relativly small differences in killing were observed for both ciprofloxaicn and levofloxacin from 8-to-24 hr. At the MPC, a -3.00 and -3.31 log killing occurred by 4 hr for ciprofloxaicn and levofloxacin, respectively. Significant killing, represented by > -3.00 log reductions in viability, was measured from 2-to-24 hr for both ciprofloxaicn and levofloxacin.

Mutant growth for the *P. aeruigonsa* strain PA-25 was defined by growth on agar plates containing 1 and 8 μg/ml of ciprofloxaicn and levofloxacin, respectively. At the MIC and MPC drug concentrations, mutant growth was detected in all culutres tested at time 0 hr. At the MIC drug concentration, mutant growth increased over 24 hr, accounting for the majority of cells present in culture, and reached the detection limit ($\geq$ + 6.00 log) of the assay by 10 and 16 hr for ciprofloxaicn and levofloxacin, respectively (Table 4.3.6). Increased killing of mutant colonies was observed for cultures treated

Table 4.3.6. Killing of *P. aeruginosa* Strain PA-25 by Ciprofloxacin and Levofloxacin at the MIC and MPC

Original Strain	Killing at the MIC (μg/ml)	Fluoroquinolone	Time (h)	Original broth concentration (CFU/ml)	Log Reduction/growth of entire culture (CFU/ml)	Log Reduction/ growth of mutant growth (CFU/ml)
PA-25	MIC_{cipro} = 0.125 MIC_{levo} = 0.5	Ciprofloxacin Levofloxacin	0	1.22×10^8 3.92×10^7	--------[a] --------[b]	+1.23[c] +2.8[d]
		Ciprofloxacin Levofloxacin	4	1.2×10^8 1.94×10^7	-0.007 -0.31	+1.23 +2.8
		Ciprofloxacin Levofloxacin	8	3.1×10^6 6.8×10^5	-1.59 -1.16	+2.28 +3.00
		Ciprofloxacin Levofloxacin	12	1.64×10^6 8.87×10^5	-1.87 -1.65	+2.73 +≥6.00
		Ciprofloxacin Levofloxacin	18	6.58×10^6 5.53×10^5	-1.27 -1.85	+≥6.00 +≥6.00
		Ciprofloxacin Levofloxacin	24	6.94×10^6 5.79×10^5	-1.25 -1.83	+≥6.00 +≥6.00
	Killing at the MPC (μg/ml)	**Fluoroquinolone**	**Time (h)**	**Original broth concentration (CFU/ml)**	**Log Reduction/growth of entire culture (CFU/ml)**	**Log Reduction/ growth of mutant growth (CFU/ml)**
PA-25	MIC_{cipro} = 2 MIC_{levo} = 16	Ciprofloxacin Levofloxacin	0	3.41×10^7 4.23×10^7	-------[c] ----------d	+1.07[c] +1.21[d]
		Ciprofloxacin Levofloxacin	4	3.38×10^4 2.06×10^4	-3.00 -3.31	+1.10 +1.29
		Ciprofloxacin Levofloxacin	8	2.38×10^4 1.7×10^4	-3.16 -3.39	+1.00 +1.32
		Ciprofloxacin Levofloxacin	12	7.65×10^4 1.03×10^4	-3.65 -4.61	+1.00 +1.00
		Ciprofloxacin Levofloxacin	18	6.2×10^3 1.84×10^2	-3.74 -5.36	+1.00 +1.00
		Ciprofloxacin Levofloxacin	24	2.85×10^2 3.44×10^1	-5.07 -6.08	+1.00 +1.00

Maximum log reduction; [a]-8.08, [b]- 7.52. [e]-7.53, [f]-7.63.
Maximum log growth; [c]+ ≥6.0, [d]+ ≥6.0.

with the MPC drug concentrations at every time point sampled. For ciprofloxaicn treated cultures, mutant colonies were recovered at 0, 2, and 4 hr (+ 1.1, 1.08, and 1.1 log growth), however, no mutants could be recoverd beyond 4 hr. For levofloxacin, mutants were recovered from 0-to-20 hr (+ 1.00 to 1.49 log growth). By 24 hr, no mutants were recoverd from ciprofloxacin- or levofloxacin-treated cultures, which represented a 5 log difference in mutant recovery when mutant growth at the MIC drug concentration was compared.

4.3.9 Ceftazidime plus Ciprofloxacin/Levofloxacin Combination MPC Results for Clinical Isolates of *P. aeruginosa*

Many of the clinical isolates tested had a levofloxacin and/or ciprofloxacin MPC value above a breakpoint for resistance, and in some cases, above a clinically achievable concentration (based on recommended doses). Synergistic activity between antimicrobial combinations, including fluoroquinolones and cephalosporins against strains of *P. aeruginosa,* have been documented *in vitro* and *in vivo* (110, 309). Fish *et al* (110) documented the successful clinical utility of fluoroquinolones used in combination with anti-pseudomonal cephalosporins for treatment of *P. aeruginosa* that demonstrated high MICs to the antimicrobial agents when tested as a single agent. I suspected that combination MPC testing could be used to restrict the selection of resistant mutants in isolates that had high fluoroquinolone MPCs. In an effort to identify antimicrobials that could be used in combination testing, I measured individual MPC results for four different classes of antimicrobials used to treat *P. aeruginosa* (Table 4.3.7). With the exception of ciprofloxacin (400 mg I.V. q8h) and possibly ceftazidime (2 g I.V.), the MPC results for all isolates tested (n=7) exceeded the peak serum concentrations for all antimicrobials tested. The MPC_{90} for clinical isolates of *P. aeruginosa* against imipenem (n=10) and ceftazidime (n=14) were $\geq$ 64 μg/ml, in both cases. For the aminoglycosides gentamicin, and tobramycin, the MPC_{90} results (n=25) were 64 and $\geq$ 64 μg/ml, respectively. MPC_{90} results for ciprofloxacin (n=155), levofloxacin (n=155) and ofloxacin (n=10) were 4, 16, and $\geq$ 64 μg/ml, respectively.

Table 4.3.7 MPC Results for Antimicrobials Against Clinical Isolates of *P. aeruginosa*

Antimicrobial Class	Antimicrobial Agent	N	MPC_{50} μg/ml	MPC_{90} μg/ml	Peak Serum Concentration C_{max} (μg/ml)	Dose	Serum (t ½ hr)	Ref
Carbapnenem	Imipenem	10	≥64	≥64	40	500 mg IV	1	(98, 127, 357)
Cephalosporin	Ceftazidime	14	32	≥64	60	1g IV	1.8	(74, 127)
					144	2g IV TID	2	(21, 127)
Aminoglycoside	Tobramycin	25	8	64	4-8	1.2 mg/kg IV	2,5	(19, 127)
	Gentamicin	25	32	≥64	4-8	1.2 mg/kg IV	2,5	(19, 127)
Fluoroquinolone	Ofloxacin	10	16	≥64	4.6	7.2 mg IV	7	(127)
	Ciprofloxacin	155	2	4	2.9	500 mg	1.2	(23, 49, 68, 127, 212)
		155	2	4	4.6	400 mg IV	4	
		155	2	4	6.3	400 mg IV q8h	2	
	Levofloxacin	155	8	16	5.2	500mg	1.7	(68, 111, 127, 274, 364)
		155	8	16	6.2	500 mg IV	7	
		155	8	16	12.1	750 mg IV	7.9	

The activities of ciprofloxacin, levofloxacin, and ceftazidime against six isolates of *P. aeruginosa* are summarized in Table 4.3.8. Ciprofloxacin and levofloxacin had generally comparable MICs against the 6 isolates of *P. aeruginosa* (ciprofloxacin MIC_{90} = 0.5 μg/ml, levofloxacin MIC_{90} = 1 μg/ml) and all were considered susceptible by NCCLS criteria (Table 4.3.9). Two isolates were resistant to ceftazidime by MIC testing (isolate numbers PA-96 MIC > 8 mg/L and PA-94 MIC > 16 mg/l). Ceftazidime plus fluoroquinolone combinations yielded a reduction in the fluoroquinolone plus ceftazidme MPC in 75% (9/12) of the isolates tested and 83% (10/12) of the isolates demonstrated a reduction in the MPC value for one of the antimicrobials.

In combination with ceftazidime, an average 1.4-fold (range 2- to 4-fold) reduction in the ciprofloxacin MPC was seen in 5/6 strains tested. The average reduction in the levofloxacin MPC values upon combination of ceftazidime concentrations was one doubling dilution (range 2- to 4-fold). For ciprofloxacin tested in combination with ceftazidime, a decrease in the ciprofloxacin MPC was seen in four strains and corresponded to an average increase in ciprofloxacin susceptibility of 1.3-fold. One isolate (PA-90) did not show any decrease in the fluoroquinolone MPC when tested in combination with ceftazidime. Isolate number 96 showed a 2-fold decrease in the ciprofloxacin MPC when tested in combination with a ceftazidime concentration of 16 μg/ml. No decrease in the levofloxacin or ceftazadime MPC was observed in this strain.

Table 4.3.8 Fluoroquinolone Ceftazidime Combination MPC Results for Clinical Isolates of *P. aeruginosa*

Isolate	MIC μg/ml			MPC μg/ml			Combination MPC μg/ml		T>MPC /24h dosing interval (h)			T>$MPC_{comboination}$ /24h[a,b,,c]	
	Cipro	Levo	Cftz	Cipro	Levo	Cftz	Cipro/ Cftz	Levo/ Cftz	T>cipro [a]	T>levo[b]	T> Cftz [,c]	Cipro/ Cftz	Levo/ Cftz
PA-101	0.125	0.5	4	2	4	8	0.5/2	2/ 2	8.5	9.4	19.1	23.25 (ceftaz=24)	18.1 (ceftaz=24)
PA-96	0.5	1	8	4	8	32	2/ 16	8/ 32	5.5	2.3	5.8	8.5 (ceftaz=12)	2.3 (ceftaz=8)
PA-98	0.125	1	4	2	4	8	1/ 2	2/2	8.5	9.4	19.1	17.5 (ceftaz=24)	18.1 (ceftaz=24)
PA-90	0.125	0.5	4	2	4	32	2/ 16	4/ 16	8.5	9.4	5.8	8.5 (ceftaz=12)	9.4 (ceftaz=12)
PA-94	0.125	0.5	16	2	4	≥64	1/ 16	4/ ≥64	8.5	9.4	0.5	12 (cipro=17.5)	0.5 (ceftaz=≤6)
ATTC 27853	0.5	1	2	2	4	16	1/ 2	2/ 2	8.5	9.4	12	17.5 (ceftaz=24)	18.1 (ceftaz=24)

Cipro=ciprofloxacin, levo=levofloxacin, cftz=ceftazadime. Estimated T>MPC based on [a]400 mg ciprofloxacin IV T.I.D (212) , [b]750 mg levofloxacin IV (203) and [b]1000mg ceftazadime IV q8h (74).

Table 4.3.9 NCCLS Breakpoints for Antimicrobials Against *P. aeruginosa*

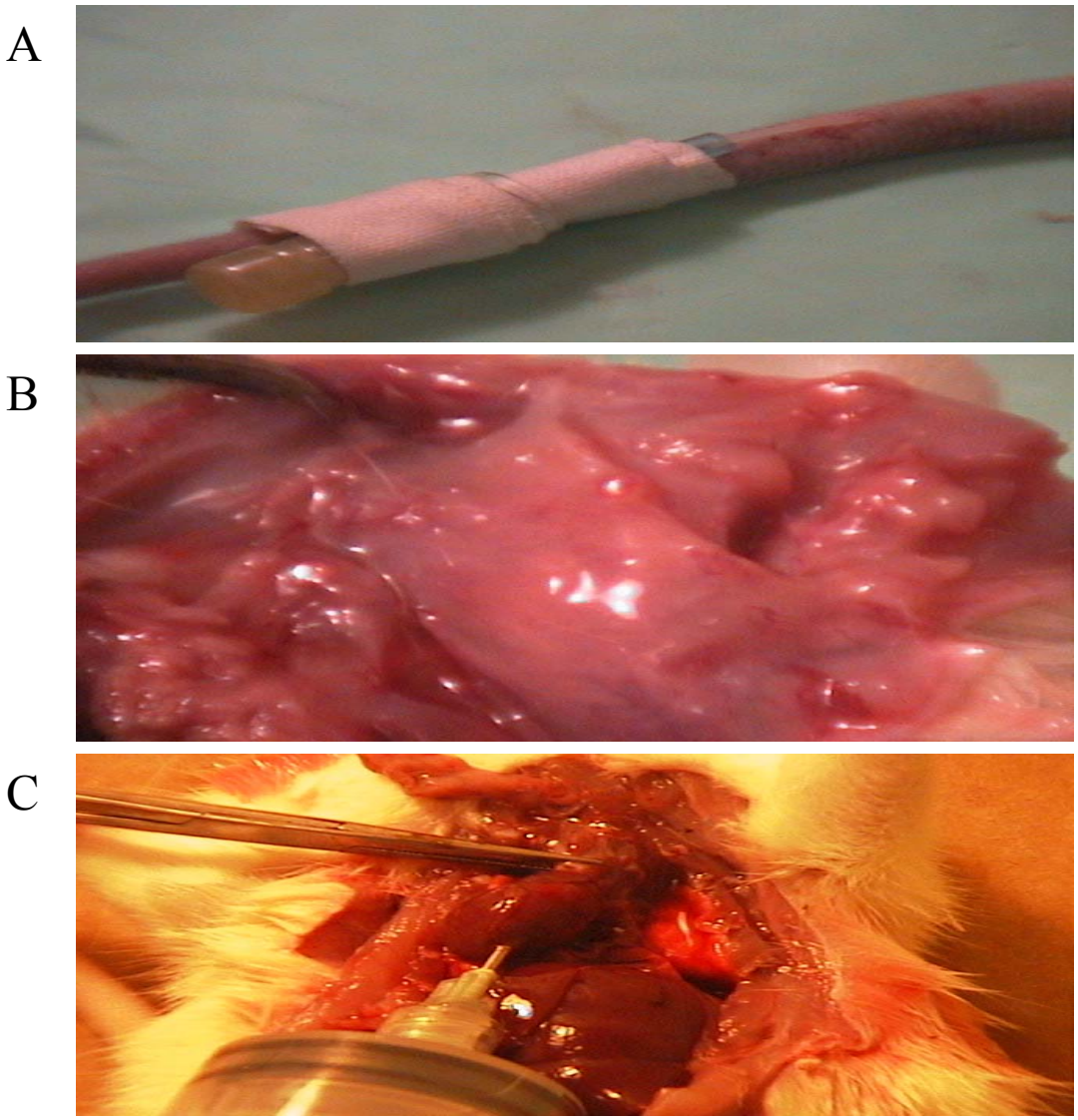
Class of antimicrobial	Antimicrobial agent	NCCLS Susceptibility breakpoints (μg/ml)		
		Susceptible	Intermediate	Resistant
Carbapnenem	Imipenem	≤ 4	8	≥ 16
3rd generation Cephalosporin	Ceftazadime	≤ 8	16	≥ 32
Aminoglycoside	Tobramycin	≤ 4	8	≥ 16
	Gentamicin	≤ 4	8	≥ 16
Fluoroquinolone	Ofloxacin	≤ 2	4	≥ 8
	Ciprofloxacin	≤ 1	2	≥ 4
	Levofloxacin	≤ 2	4	≥ 8

Ceftazidime plus fluoroquinolone combinations resulted in a reduction in the MPC, to at least one of the agents used in combination, for 6/6 strains tested by ceftazidime plus ciprofloxacin and 4/6 strains tested against ceftazidime plus levofloxacin concentrations. Susceptibility, as determined by a susceptible NCCLS breakpoints was restored in 4/6 strains tested by ceftazidime plus ciprofloxacin. Similarly, ceftazidime plus levofloxacin restored susceptibility in the same 3/6 strains tested. Combination MPC results increased the length of time ceftazidime and ciprofloxacin combinations are projected to exceed the combination MPC result by 6.5 and 7.5 hr (based on 400 mg T.I.D. ciprofloxacin, 1 g ceftazidime). Levofloxacin and ceftazidime combinations resulted in an average increase of 1.45 hr for levofloxacin and 3.48 hr for ceftazidime in the time MPC concentrations are expected to be maintained during dosing (based on 400 mg T.I.D. ciprofloxacin, 1g ceftazidime). Additionally, at least one of the antimicrobial agents tested was in excess of the MPC for 6 hr of a potential 24 hr dosing period (Table 4.3.9). Thus, combination MPC testing with a fluoroquinolone and Ceftazidime resulted in a lower fluoroquinolone MPC (when compared to initial MPC result) in at least half of the strains tested, and demonstrated that combination MPC testing can be used to minimize the potential for selecting resistant organisms with antimicrobials that have high individual fluoroquinolone MPCs.

4.3.10 Recovery of Resistant Mutants in a Rat Abscess Model of Infection with *P. aeruginosa*

The majority of data pertaining to the MPC measurement has been obtained using *in vitro* studies, and animal models are now required to examine the clinical utility of the MPC measurement and the concept of the MSW. I used an absess rat model to create infections using $4.5x10^9$ to $3.8x10^{10}$ CFU/ml cultures of *P. aeruginosa* (Figure 4.3.11). The ciprofloxacin and levofloxacin MIC for isolate CBRH 25138 used to create infection were 1 and 2 μg/ml, respectively. The ciprofloxacin and levofloxacin MPC were 4 and 16 μg/ml. Four experimental groups consisted of rats given, 400 mg I.V. once daily dose of ciprofloxacin (400 mg I.V. (n=2) and 400 mg I.V. qh8 (n=2) and 750 mg I.V. once daily dose of levofloxacin (750 mg I.V. once daily (n=3) and 750 mg I.V. qh8 (n=2), results are summarized in Table 4.3.10. *P. aeruginosa* was recovered from infected tissues and blood samples which were were analysed for changes in susceptibility based on the MIC. Resistant mutants were defined by a $\geq$ 2-fold change in the ciprofloxacin and levofloxacin MIC relative to the MIC of the wild-type strain. Resistant organisms were recovered in 33% (3/9) of the rats tested. Of the 23 cultures recovered (total swabs plus tissues plus blood cultures), 22% (5/23) demonstrated elevated susceptibilities to both ciprofloxacin and levofloxacin. Swabs of infected tissues and homogenized tissue samples collected from a rat given a 400 mg I.V. once daily ciprofloxacin (rat number 48), grew *P. aeruginosa* with a ciprofloxacin MIC of 8 μg/ml and a levofloxacin MIC of 16 μg/ml. Swabs of infected tissues taken from rat

Figure 4.3.11 Administration of Fluoroquinolones and Collection of Clinical Samples in a Rat Abscess Model of *P. aeruginosa* Infection



Collection of infected tissue and blood samples from infected rats. Antimicrobials were administered and blood samples taken via a rat tail vein catheter (panel A). Swabs of infected tissues and infected tissues were recovered from infected rats (panel B). The circulatory system was infused with sterile saline and blood samples were collected (panel C).

Table 4.3.10 Recovery of *P. aeruginosa* from a Rat Abscess Model of Infection

Culture ID	Rat ID	Fluoroquinolone dose I.V (mg)				Infectious dose (CFU/.45ml)	Source of isolation	$MIC_{recovered}$ (μg/ml)		MIC_{parent} (μg/ml		MPC_{parent} (μg//ml)	
		Cipro 400	Cipro 400 T.I.D	Levo 750	Levo 750 T.I.D			Cipro	levo	Cipro	Levo	Cipro	Levo
1	47			+		3.9×10^{10}	tissue	2	4	2	4	4	16
2				+			swab	2	4				
3				+			swab	2	4				
4				+			blood	2	4				
5				+			blood	2	4				
6				+			tissue	2	4				
7	48	+					tissue	8	16				
8		+					swab	8	16				
9		+					swab	8	16				
10		+					blood	2	4				
11		+					blood	2	4				
12		+					tissue	2	4				
13	49			+			Tissue	2	4				

Culture ID	Rat ID	Fluoroquinolone dose I.V (mg)				Infectious dose (CFU/.45ml)	Source of isolation	$MIC_{recovered}$ (μg/ml)		MIC_{parent} (μg/ml		MPC_{parent} (μg//ml)	
		Cipro 400	Cipro 400 T.I.D	Levo 750	Levo 750 T.I.D		tissue	Cipro	levo	Cipro	Levo	Cipro	Levo
14	49			+		3.9×10^{10}	swab	2	4	2	4	4	16
15	56		+			4.5×10^{9}	swab	2	4				
16	50	+				3.9×10^{10}	swab	2	4				
17		+					swab	2	4				
18		+					blood	8	32				
19		+					blood	2	4				
20		+					tissue	2	4				
21	51			+		4.5×10^{9}	swab	8	16				
22				+			blood	2	4				
23	59	Control (no drug)				3.9×10^{10}	swab	2	4				
24	60	Control (no drug)				4.5×10^{9}	swab	2	4				
25	53				+		swab	2	4				
26	55				+		swab	2	4				
27	58		+				swab	2	4				

Nine rats, divided into 4 experimental groups; 1.) ciprofloxacin dosed 400 mg I.V, 2.) Ciprofloxacin dosed 400 mg I.V T.I.D., 3.) Levofloxacin dosed 750 mg I.V, and 4.) Levofloxacin dosed 750 mg I.V. T.I.D were tested. Plus signs indicate s the recoverey of *P. aeruginosa* from treated animals

number 51 (culture 21) which had been given a 750 mg I.V. once daily levofloxacin dose grew resistant *P. aeruginosa* with a ciprofloxacin and levofloxacin MIC of 8 and 16 μg/ml, respectively, which correlated with the MPC of the original parental strain. Blood samples taken from a second rat (rat number 50, culture number 18) which had been given a 400 mg I.V once daily dose of ciprofloxacin grew resistant *P.aeruginosa* with a ciprofloxacin and levofloxacin MIC of 8 and 32 μg/ml, respectively. The MIC of the recovered culture (culture # 18) showed a 4- and 6-fold increase in the MICs to ciprofloxacin and levofloxacin. No mutants were recovered in rats, which had been given a three times daily dose. Overall, mutants demonstrated a 4-fold decrease in fluoqouinolone susceptibility in 33% (3/9) of the rats tested which demonstrated that fluoroquinolone-resistant mutants could be readily selected and enriched under fluoroquinolone concentrations which fail to inhibit the most resistant mutant present in 10^{10} CFU/ml cultures of *P. aeruginosa*.

4.4 DISCUSSION

Fluoroquinolone development has resulted in more potent agents such as moxifloxacin, gatifloxacin and gemifloxacin, however, these agents do not exhibit enhanced activity against *P. aeruginosa.* Consequently, maintaining the efficacy of existing fluoroquinolone agents with activity against *P. aeruginosa,* such as ciprofloxacin and levofloxacin, is of paramount importance. Conventional susceptibility considerations are inadequate for choosing between ciprofloxacin and levofloxacin for treatment of infections caused by *P. aeruginosa:* resistance develops frequently to both and neither compound demonstrates a clear advantage when surveillance data, *in vitro* activities, and drug PK are considered collectively (212, 225, 323). Nevertheless, clinical surveys indicate that increased prevalence of resistance is associated with a switch from ciprofloxacin to ofloxacin/levofloxacin (22, 283, 303). When ciprofloxacin and levofloxacin were compared for activities against cultures containing subpopulations of resistant mutants, ciprofloxacin was more active by two criteria. First, MPC, a value that approximates the MIC of the least susceptible mutant, was three to four times higher for levofloxacin, both by population analysis (Figure 4.3.1) and by survey of more than 150 clinical isolates (Figure 4.3.4). Second, larger numbers of moderately susceptible and high level resistant mutants were more likely to be recovered following exposure to levofloxacin than ciprofloxacin (Figure 4.3.1), particularly with clinical isolates having intermediate fluoroquinolone susceptibility (Figure 4.3.2).

When fluoroquinolone-susceptible clinical isolates of *P. aeruginosa (*n=6*)* were applied to agar plates containing various concentrations of ciprofloxacin or levofloxacin, recovery of colonies decreased as the fluoroquinolone concentration approximated the MIC (Figure 4.3.1). In these experiments, each data point represented a 10^{10} CFU/ml culture exposed to defined ciprofloxacin or levofloxacin drug concentrations. At higher concentration, both compounds exhibited a pronounced inflection in mutant recovery, visualized as a generalized plateau regions at concentrations above the MIC. As fluoroquinolone concentrations increased, a second decline in CFU corresponding to the inhibition of first-step mutants was seen for both ciprofloxacin and levofloxacin, and eventually a concentration of ciprofloxacin and levofloxacin was reached which prevented the growth of the most-resistant first-step mutant present in a population of $\geq 10^9$ cells. For ciprofloxacin and levofloxacin, MPC drug concentrations were measured at 3 and 9.5 μg/ml, respectively. Comparison of the MSWs for ciprofloxacin and levofloxacin indicated that levofloxacin was 1500 times more efficient at selecting resistant mutants. The quantitative differences in mutants selected by ciprofloxacin and levofloxacin extend beyond the conventional understanding of fluoroquinolone activity for *P. aeruginosa.* For example, differences in the anti-pseudomonal activities between ciprofloxacin and levofloxacin manifest in an approximate 2-fold difference in the MIC. If differences in mutant selection between ciprofloxacin and levofloxacin parallel activity described by MIC measurements, then one should expect parallel activity against first-step resistant

mutants, albeit shifted by a factor of 2-fold toward higher levofloxacin drug concentrations. As expected, this relationship holds true for 10^5 to 10^6 cells present in 10^{10} CFU/ml cultures, however, parallel fluoroquinolone activity was not seen when mutant recovery at concentrations above the MIC were examined. Quantitative differences in mutant selection were determined by analyzing the area created under respective ciprofloxacin and levofloxacin MSWs. Levofloxacin selected more mutants, at higher concentrations, and over wider concentration ranges when compared to ciprofloxacin which could be expressed in a ciprofloxacin: levofloxacin ratio of 1:1500 ($\geq$ 3 log difference in CFUs). I also examined an isolate of *P. aeruginosa* that exhibited intermediate fluoroquinolone susceptibility (MIC = 2 and 4 μg/ml for ciprofloxacin and levofloxacin, respectively). At moderate drug concentrations (0.5-to-8 μg/ml), resistant mutants were more frequently recovered from agar containing concentrations of levofloxacin (Figure 4.3.1) Thus, the preferential ability of ciprofloxacin to restrict mutant growth is also evident with isolates having intermediate susceptibility.

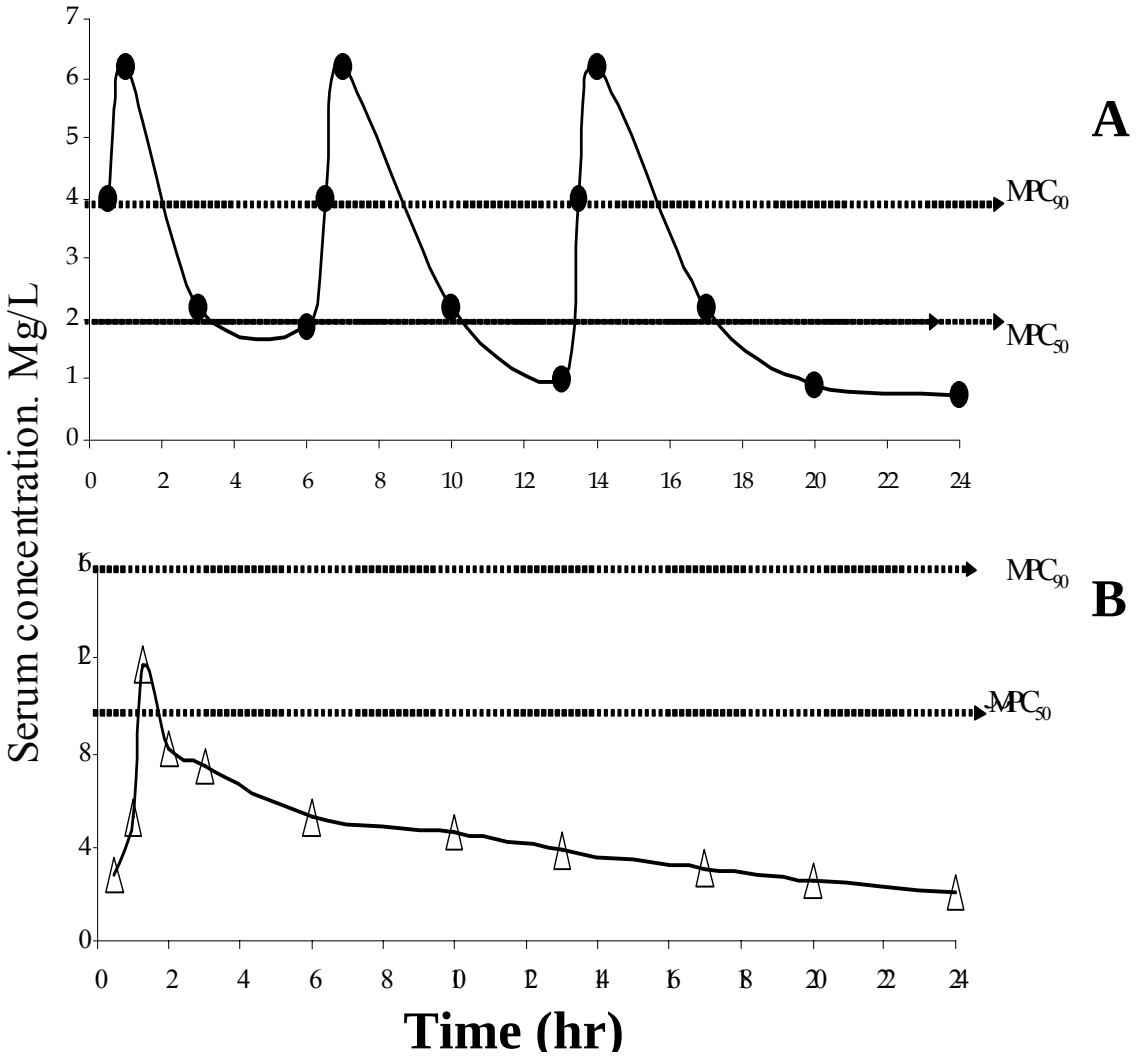
Adapting MPC testing to incorporate conventional 2-fold dilution agar testing resulted in modal MPC values of 2 and 8 μg/ml for ciprofloxacin and levofloxacin and MPC_{90} of 4 μg/ml and 16 μg/ml, respectively, when 155 clinical isolates of *P. aeruginosa* were tested. Thus, the upper boundary of the mutant selection window (i.e., the MPC) was about 4 times higher for levofloxacin. The significance of this finding can be reflected by examining the percentage of isolates whose MPC value fell at or above a fluoroquinolone resistant breakpoint. When the MPC results for ciprofloxacin

and levofloxacin were analysed in relation to NCCLS described breakpoints for resistance, the number of isolates that showed resistance to levofloxacin (67%) were nearly twice the number that demonstrated resistance to ciprofloxacin (36%) (Figure 4.3.6). High-level resistance assessed by MPC values of $\geq$16 μg/ml, correlate with poor clinical success (124) and were selected by levofloxacin in 13% of the isolates compared to 5% for ciprofloxacin.

The significance between ciprofloxacin and levofloxacin MPC results remains an area of intense investigation. A proposed model of infection suggests that higher numbers of mutants selected under levofloxacin dosing places the patient at increased risk for enriching mutant populations. Comparison of the MSW for ciprofloxacin and levofloxacin revealed that the levofloxacin MSW was twice as large as the MSW for ciprofloxacin. This difference is not reflected in current MIC testing for susceptibility.

Comparisons between compounds, based on the MPC, require consideration of drug PK. I have suggested comparisons based on the time relevant drug concentrations are above the MPC (41, 149), since during this time mutant growth will be restricted. Levofloxacin concentrations failed to exceed the MPC_{90} value for any portion of the dosing interval (based on 750 mg I.V. once daily), while concentrations of ciprofloxacin are projected to remain in excess of the MPC_{90} for approximately 5.5 of a 24 hr dosing interval (based on 400 mg T.I.D., I.V.) (57, 212, 274) (Figure 4.4.1). For some antibiotic dosages, the drug-susceptible population may completely replaced with resistant mutants over time if the MPC is not targeted throughout dosing, or achieved

Figure 4.4.1 Relationship of MPC Results to Ciprofloxacin and Levofloxacin Dose Response Profiles



The relationship between MPC_{90} and MPC_{50} results for 155 clinical isolates of *P. aeruginosa* and the ciprofloxacin (400 mg I.V. T.I.D) dose response curve is shown in A. The ciprofloxacin dose-response curves were adapted from Lipman *et al* (212). B shows the relationship between MPC_{90} and MPC_{50} results for 155 clinical isolates of *P. aeruginosa* and the levofloxacin (750 mg I.V.) dose response curve. Data for levofloxacin was adapted from product insert (274) ciprofloxacin (400 mg I.V. T.I.D) levaquin package insert (274).

for sufficient time to inhibit mutant growth. Clinically, this may manifest in failure of an infection to respond to therapy or a relapse of infection with drug-resistant mutants shortly after a prescribed course of antibiotic therapy is completed. Another approach is to consider the time that the two compounds are inside the mutant selection window, since longer times are expected to facilitate mutant enrichment (14, 86, 109). Here the difference is not striking: 100% for levofloxacin and 80% for ciprofloxacin (Figure 4.4.1). However, these numbers fail to consider differences in selection of low and moderately resistant mutants. For ciprofloxacin, 65% of the isolates tested were inhibited at concentrations $\leq$2 μg/ml compared to 30% which were inhibited at levofloxacin concentrations $\leq$4 μg/ml. Thus, for low-level resistant mutants, ciprofloxacin concentrations would remain in the window for 44% of each 24 hr-period (based on a T.I.D. dose), while levofloxacin concentrations would be in the window for 80% of the 24 hr period (based on once daily dosing).

Fluoroquinolone resistance in *P. aeruginosa* has been described for strains demonstrating MICs of $\geq$8 μg/ml to both ciprofloxacin and levofloxacin and these strains frequently contain *gyrA* mutations at sites 83 and 87 (4). Comparison of MPC values demonstrated that 8% of the mutants selected by ciproflxoaicn concentrations had a MPC value $\geq$8 μg/ml compared to 67% of the strains tested which had a levofloxacin MPC value of $\geq$ 8 μg/ml (Figure 4.4.2). *In vitro* studies have shown that acquisition of *gyrA* mutations in Gram-negative bacteria facilitate the step-wise selection of additional target mutations which confer higher degrees of fluoroquinolone

resistance (256, 267, 307, 342). The selection of a first-step quinolone target mutation also increases the frequency with which additional mutations are selected relative to strains that do not harbour gyrase or topoisomerase mutations (207). Sequence analysis of >400 base pair regions of the *gyrA* and *parC* genes demonstrated that selected mutants of *P. aeruiginosa* contain QRDR and as well as target mutations which map outside of the characterized QRDR regions of *gyrA* and *parC*. Mutants selected from 11 different strains of *P. aeruiginosa* were analyzed and five mutants contained a *gyrA* T83I mutation which has been proven to contribute to fluoroqouinolone resistance based on genetics studies (2). Additional mutants contained uncharacterized mutations in regions of the *gyrA* and *parC* genes that were not present in the parental strains, suggesting a possible association between target mutations and phenotypic resistance. Examination of mutants recovered from mutant selection curves revealed that low concentrations of fluoroquinolone selected only non-*gyrA* mutants; at slightly higher concentrations, *gyrA* and *parC* variants were detected. Failure to detect target mutants at low concentrations is likely due to the overwhelming numbers of susceptible cells relative to target mutants, or may be attributed to the presence of efflux mechanisms which are known to operate in clinical isolates of *P. aeruginosa* (176, 205, 219). However, overexpressions of different efflux proteins in the selected mutants were not analyzed in this study. High fluoroquinolone concentrations selected T83I *gyrA* mutants, which were readily detected in isolates with intermediate fluoroquinolone

Figure 4.4.2. Stratification of the Mutant Selection Window Based on Ciprofloxacin and Levofloxacin MPC Distributions for 155 Clinical Isolates of *P. aeruginosa*

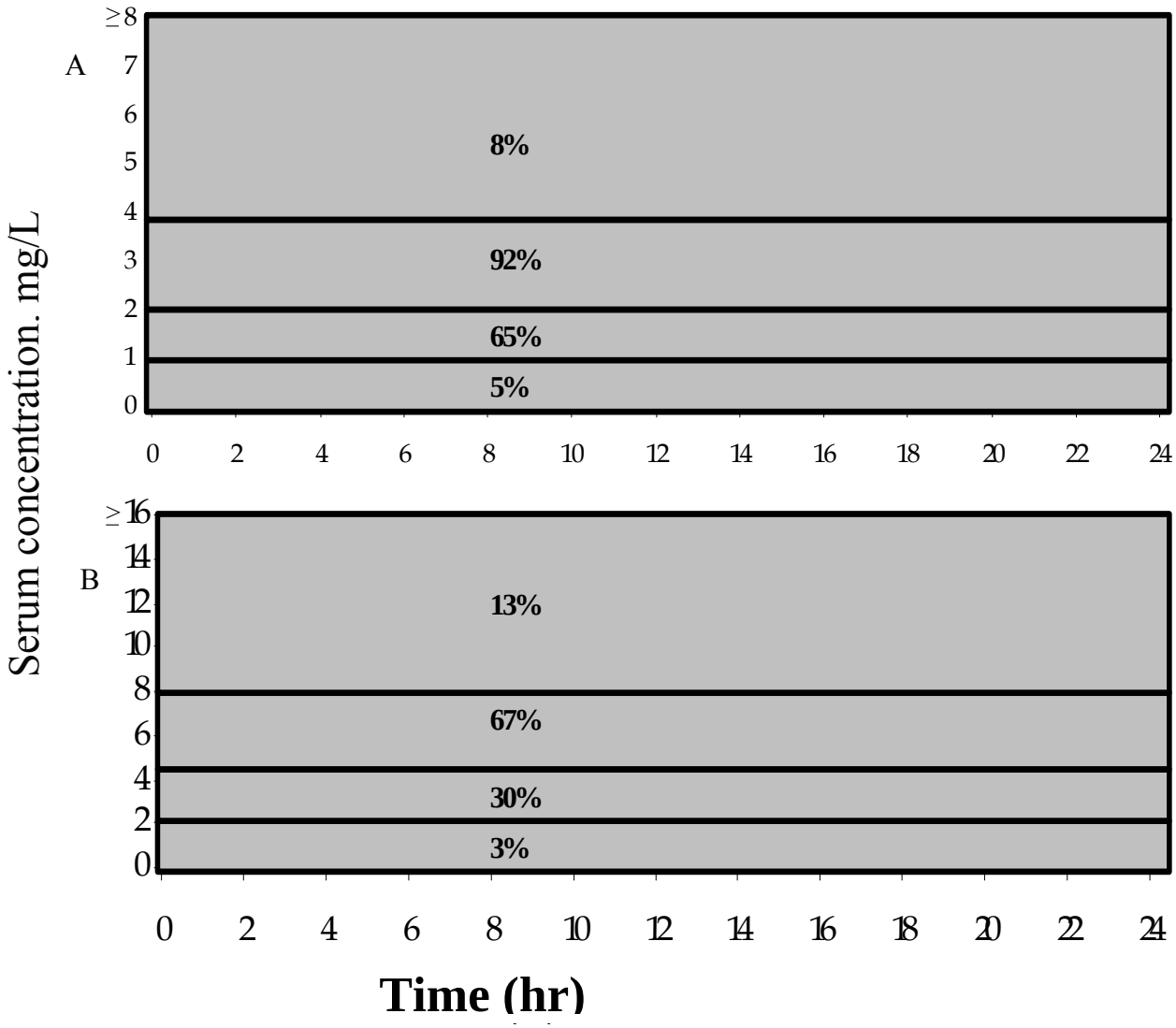


Figure A shows **t**he percentage of isolates with ciprofloxacin MPC values of ≤ 1, ≤ 2, ≤ 4, and ≥ 4 μg/ml and the projected time ciprofloxacin concentrations are expected be within the MSW for each group of isolates. Figure B shows **t**he percentage of isolates with ciprofloxacin MPC values of ≤ 2, ≤ 4, ≤ 8 and ≥ 8 μg/ml and the projected time levofloxaicn concentrations are expected be within the MSW for each sub-group of isolates. Ciprofloxaicn concentratins based on a 400 mg I.V. T.I.D. dose (212). PK profile for levofloxacin based on published reports and product information (274).

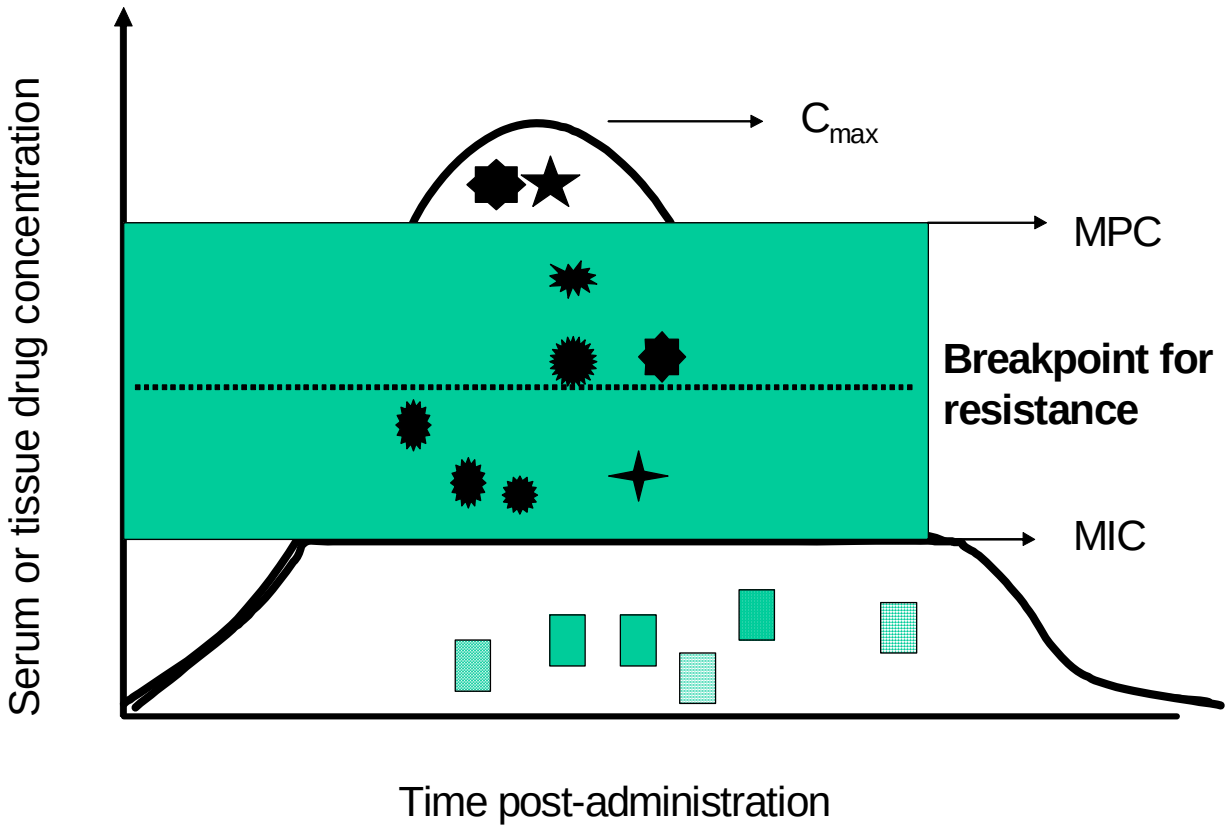
susceptibilities, suggesting that isolates with intermediate phenotypes appear to select the T83I mutation as the "next-step" mutant in the progression of fluoroquinolone resistance. This suggestion implies that the T83I mutant(s) should be present in $\geq 10^{10}$ CFU/ml cultures of *P. aeruginosa* with intermediate susceptibility to both ciprofloxacin and levofloxacin, and should be selected by both ciprofloxacin and levofloxacin drug concentrations. For some strains, no target mutants (i.e., *gyrA/parC*) could be detected in colonies selected from ciprofloxacin plates. Two related factors may contribute to this finding. 1.) Sequence analysis was preformed on a limited number of mutants, thus, target mutants present in low numbers, may not have been detected in the limited numbers of colonies sampled. 2.) Differences in mutant recovery may reflect differences in bactericidal activity which are not reflected in the MPC measurement. Thus, enhanced killing of mutant cells at low and moderate concentrations of ciprofloxacin may reduce, or eliminate the T83I mutant population, making it difficult to detect target mutants relative to levofloxacin.

Step-wise acquisition of resistance may explain why some of the mutations identified in recovered mutants of *P. aeruginosa* have yet to be reported in the literature, because first-step mutants selected from susceptible strains produce small changes in susceptibility that are not detected as a 2-fold change in susceptibility (i.e., the MIC). Additionally, resistance is not an absolute term and many levels of resistance can be attained through acquisition of target mutations. For example, over 30 different resistance alleles have been obtained with fluoroquinolones and *M. tuberculosis* (332,

334). Analysis of *P. aeruginosa* mutants identified 10 different *gyrA*, and 8 *parC* mutations. However, "resistance" is typically detected and acknowledged in a 2-fold change in the MIC and would not account for target mutations that may lead to the selection of mutants with higher degrees of resistance. In this respect, the definition of the MPC should include the concentration which prevents the growth of the most resistant phenotype selected from within a given susceptible population. This hypothesis may also help to explain detection of multiple target mutations in some of the recovered mutants. Figure 4.4.3 represents a potential dose response curve and mutant selection window for a hypothetical fluoroquinolone antimicrobial. For any given pathogen, different susceptible phenotypes are likely to exist, each conferring a relative degree of "resistance" which manifest through differences in susceptible MICs. Thus, a number of different susceptible strains can exist within a population of cells classified as susceptible. Allelic diversity within the regions of the *gyrA* and *parC* genes coupled with efflux pump expression may influence the specific resistance determinant selected. A given compound may only be able to inhibit the selection of a defined number of mutants.

Therefore, once a certain degree of "resistance" is achieved in a parental strain, the "next-step" mutant select may not be inhibited (or killed) by clinically relevant concentrations. Differences between an agent's propensity to select for resistance, as determined by the MPC, may reflect targeted activities against a defined number an/or variants of first-step resistant mutants selected from within genetically distinct

Figure 4.4.3 Relationship Between Selection of Resistant Mutants and Initial Degree of Susceptibility



A proposed model for mutant selection suggests that differences in allelic variants selected in 10^{10} CFU/ml cultures is dependent on the degree of susceptibility in initial susceptible populations. Different susceptible isolates are represented as rectangles, all of which confer a different degree of susceptibility as determined by variance in susceptible MIC values. Mutants selected from 10^{10} CFU/ml cultures of susceptible isolates are illustrated by stars. For any given fluoroquinolone agent, the growth of a finite number of mutants can be inhibited at concentrations below a breakpoint for resistance for the drug. Thus, differences in the MPC results between antimicrobials of the same class may relate to the preferential ability of an agent to inhibit a larger proportion of the mutants selected from within susceptible populations.

susceptible populations. Therefore, differences between ciprofloxacin and levofloxacin MPC results may be due to the preferential ability of ciprofloxacin to inhibit the growth of a larger number of first-step mutant populations that are selected from within a larger array of susceptible isolates. The *gyrB* and *parE* subunits of DNA gyrase and topoisomerase IV are secondary quinolone targets and resistance and *gyrB* and *parE* mutations have been found in strains of *P. aeruginosa* which already contain *gyrA* or *gyrA* + *parC* mutations (156, 200, 248).

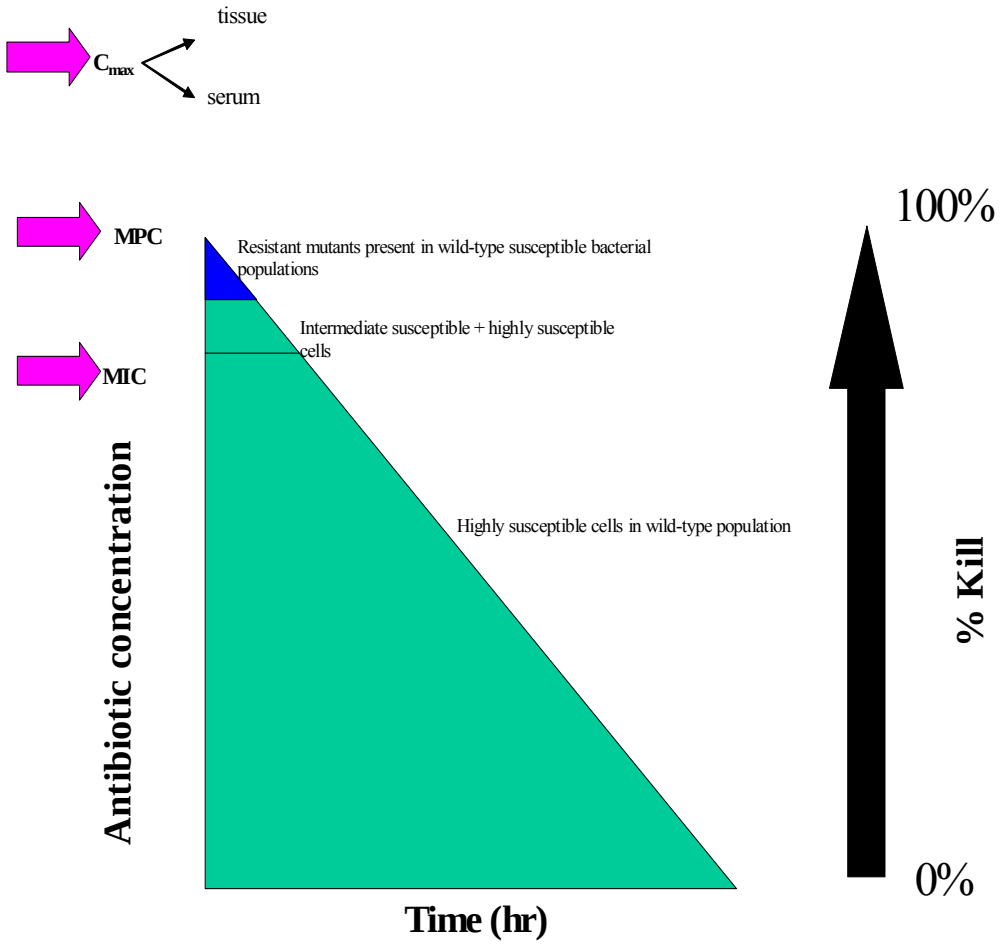
Mutations in *gyrB* and *parC* regions were not examined in my analysis as it is well known that *gyrA* mutations are the first target mutations to be obtained in Gram-negative bacteria and those *parC* mutations are secondary. Since my study looked at first-step mutations, it was not necessary to look at *parE* and *gyrB* mutations because they are unlikely to be present, but I cannot exclude the possibility that mutations in regions of the *parE* and *gyrB* gene may have contributed to resistance in some of the recovered mutants. The effect of target mutations on the subsequent development of resistance is still under investigation. MPC measurements preformed on recovered mutants will help to identify if selected mutants increase the acquisition or frequency of additional resistance alleles relative to parental colonies. MPC results for isolates with intermediate susceptibilities to ciprofloxacin and levofloxacin reproducibly selected T83I *gyrA* mutations (Table 4.3.3) suggesting that the "next-step" mutant selected from strains with intermediate susceptibility is one which confers a level of resistance that is associated with poor clinical success (4, 124).

MICs for recovered mutants ($MIC_{recovered}$) were 6- to 10-fold higher when compared to parental MICs **(**Table 5.3.2). Overall, 81% (165/204) of the recovered mutants demonstrated elevated MICs to ciprofloxacin and levofloxacin when compared to the MIC of the parental isolates and 69% (140/204) of the mutants tested had a resistant phenotype. One hundred and eight mutant colonies were recovered from ciprofloxacin concentrations above the MPC during MPC testing and 91% (98/108) of the recovered mutants were within one doubling dilution of the MPC results when re-tested by conventional MIC testing. For levofloxacin, 94% (91/96) of mutants recovered from levofloxacin plates were with one doubling dilution of the MPC measurement. Thus, mutant colonies represent stable phenotypes with decreased fluoroquinolone susceptibilities. A 50 mM solution of CCCP was added to susceptibility panels to examine whether efflux mechanisms contribute to fluoroquinolone resistance in recovered mutants (287, 365). For ciprofloxacin, 23% of the strains tested showed at least a 2-fold decrease in the $MIC_{recovered}$ in the presence of a 50 mM solution of CCCP. For levofloxacin, 16% of the strains demonstrated a $\geq$2-fold decrease in susceptibility upon the addition of CCCP. Generally, a >2- fold change in the MIC with the addition of CCCP is required to identify active efflux as a mechanism of fluoroquinolone resistance in isolates of *P. aeruginosa* (365). Thus, by this system, efflux mechanisms could not be identified as primary mechanisms of fluoroqouinolone resistance in recovered mutants of *P. aeruignsoa*, however, a lack of clear correlation between CCCP-promoted increases in fluoroquinolone accumulation and resulting

decreases in the MIC is known to exist in strains where efflux has been shown (119, 266). In addition, these studies did not account for cumulative differences in efflux expression between 10^5 and 10^{10} CFU/ml. As such, demonstration of CCCP-enhanced MIC results cannot be the sole determinant used to support (or disprove) the role of efflux mechanisms in fluoroquinolone resistance. PFGE of recovered mutants and parental isolates showed no differences in the DNA banding profiles (Figure 4.3.7) and indicated that recovered mutants did not represent distinct clones relative to parental organisms but rather were selected from within parental populations.

Concentration-dependent killing by fluoroquinolones suggests that targeting MPC in antibiotic dosing strategies may have a profound impact on minimizing resistance and the speed to clinical resolution (34, 146). Thus, agents whose serum/tissue concentrations do not exceed the MPC for the entire length of the dosing interval may still be effective at minimizing the selection of resistant mutants if mutant killing occurs within a time interval during which fluoroquinolone concentrations exceeds the MPC (Figure 4.4.4). Conventional kill-curves performed with fluoroquinolones do not reflect the killing of first-step resistant mutants because the inoculums used are too low to account for their presence. Killing at the MIC drug concentrations resulted in < -2 log reductions in overall viability for both isolates tested. Mutant outgrowth was observed in both ciprofloxacin and levofloxacin treated cultures which accounted for the majority of the cells present in cultures by 12-24 hr. Killing at the MPC drug concentration resulted in $\geq$ -3 log killing between 4 and 8 hr and mutant

Figure 4.4.4 The Relationship Between Bacterial Killing and the MPC for Fluoroquinolones



Hansen & Blondeau 2001

Mutant subpopulations are likely to exist in large inocula that require MPC concentration to inhibit their growth. The width of the killing triangle is determined by the time required to inhibit both susceptible and first-step resistant cells, represented in hrs. Fluoroquinolone activity and killing do not always parallel, therefore, a narrow killing triangle may represent an antimicrobial agent that possess lower intrinsic activity (as assessed by a high MIC) against a specific pathogen, but may display increased killing against first-step resistant mutants.

growth was restricted. Thus, killing at the MPC drug concentration resulted in $\geq$ 5 log difference in mutant killing compared to killing at the MIC drug concentration when the contents of 10^{10} CFU/ml cultures of *P. aeruginosa* were tested against ciprofloxacin and levofloxacin. A difference of ~4 logs growth was observed between cultures treated with MIC and MPC fluoroquinolone concentrations indicating that killing at the MPC drug concentration resulted in rapid reductions in both susceptible and mutant cells which is hypothesized to decrease the potential for selecting resistant mutants and positively impact on clinical outcome since clinical performance is dependent on antibiotic-mediated bacterial eradication in a number of infections (67).

Recently, Firsov *et al* (109) demonstrated that fluoroquinolone concentrations which fluctuated within the MSW were associated with a decrease in the susceptibility of *S. aureus* to fluoroquinolones. Killing experiments based on the MIC drug concentration failed to achieve significant bacterial killing and the outgrowth of mutant subpopulations paralled the decline in growth of the susceptible population. Killing experiments conducted at the MPC drug concentration using 10^{10} CFU/ml cultures provide preliminary insight into the relationship between drug PK and time required for mutant killing. For ciprofloxacin, the projected time serum drug concentrations are expected to in excess of the MPC_{90} for *P. aeruignsoa* is estimated to be 5.5 hr for 90% of strains and 13 hr for organisms with ciprofloxaicn MPCs of $\leq$2 μg/ml. For levofloxacin, the MPC_{90} was 16 μg/ml and the modal MPC value was 8 μg/ml. As such, no current recommended dosage of levofloxacin would exceed 16

μg/ml and for organisms with MPCs of 8 μg/ml, serum drug concentrations would exceed this value for approximately 2 hr (144, 146, 148). Serum drug concentrations for levofloxacin fall within the experimentally defined MSW for a greater time and correlate with decreased time above the MPC required to prevent the selection and enrichment of resistant subpopulations. Using an *in vitro* PK/PD model to simulate fluoroquinolone doses that fluctuated within the MSW for *S. aeurus,* Firsov *et al* (109) demonstrated that drug concentrations which remain within the MSW for ≥45% of the dosing interval could readily select for resistant organisms. It can be further argued that the practical role of bacterial killing on enrichment of resistant mutants within the MSW serves to minimize the rounds of replication for mutant cells. To date, the anti-infective community relates the concentrations of antimicrobial agents in plasma to the MICs for relevant pathogens and thereby makes recommendations on antibiotic dosages and dosing intervals. Thus, it may be argued that the relevant concentration used in *in vitro* kill kinetic experiments should include the relevant serum/tissue Cmax. This argument has been the basis for debate in the literature regarding the bactericidal action of ciprofloxacin and levofloxacin against *P. aeruginosa* (323). Levofloxacin is dosed based on tolerability and, as such, accumulates in the serum and infected tissues to a much greater degree than can be obtained for ciprofloxacin. This allows levofloxacin to be dosed at much higher levels, thereby achieving a larger AUC/MIC for a single dose which may result in higher rates of bacterial killing, as the action of fluoroquinolones is concentration dependent. Thus, recent reports citing superior killing by levofloxacin

against *P. aeruginosa* have fuelled the debate over appropriate quinolone use in infection caused by this agent (130, 225, 322, 323).

Gillespie *et al* (130) examined the mutation rate for ciproflxoaicn and levofloxacin against strains of *P. aeruignsoa*, using large inocula. In the 11 examples where the mutation rates could be directly compared, levofloxacin was less likely to select resistant mutants in five instances. Mutants recovered from six strains were tested for killing by ciprofloxacin and levofloxacin at the expected pulmonary epithelial lining fluid drug concentration and no consistently significant differences in killing between the two agents was observed, despite the fact that levofloxacin concentrations represent a 2-fold higher epithelial lining fluid drug concentration than for ciprofloxacin. In the context of my kill experiments, some notable observations in the Gillespie study require further explanation. The inoculums used to select mutants ranged from $6.6x10^6$ to $3 x10^9$ CFU/ml For levofloxacin, 1/7 stains tested utilized a test inoculum $\geq 10^9$ CFU/ml and only 2 of the strains tested were exposed to inoculums $>10^8$ CFU/ml. Previous experiments have demonstrated that a minimum of 10^9 CFU/ml is required to consistently detect sub-populations of first step resistant cells (146), thus the absence of mutants selected by levofloxacin may be a result of using inoculums that do not contain mutants (or high enough numbers of mutants) to be detected. In some cases, the authors failed to acknowledge that direct comparisons between the mutant selection potential for ciprofloxacin and levofloxacin were made using inocula that differed up to 1000-fold. In addition, many of the strains used in the study represented

intermediate phenotypes. For fluoroquinolones and *P. aeruginosa*, application of MPC may only be significant in the context of fully susceptible strains. Therefore, their conclusion that levofloxacin is less likely than ciprofloxacin to select resistant mutants may not hold true when susceptible strains are used and when inocula of $>10^9$ CFU/ml are tested.

Additional reports have also described equivalent killing of ciprofloxacin and levofloxacin against *P. aeruginosa* (271, 362). MacGowan *et al* (225) further qualified the differences in killing by suggesting that the bactericidal activities of levofloxacin and ciprofloxacin are equivalent provided similar AUC/MIC ratios can be obtained. Thus, apparent differences in the bacteristatic and bactericidal activities of ciprofloxacin and levofloxacin may relate to the initial degree of resistance present in the parental isolate. As has been previously implied, it is possible that once a defined degree of resistance is achieved, the bacteristatic and bactericidal activity of ciprofloxacin and/or levofloxacin may be compromised to the point where clinically achievable concentrations are unable to control the mutant either through inhibition of growth or bacterial killing. Although levofloxacin is able to achieve a high Cmax (12.1 μg/ml) (274), it is expected to remain in excess of the MPC_{90} and MPC_{50} for 0 and 3 hr, respectively, of the dosing interval for *P. aeruginosa*. Some strains of *P. aeruginosa* possess MPCs which lie outside clinically achievable concentrations. Enhanced killing related to a high Cmax may be offset by the fact that relevant killing concentrations are attained for short periods. Serum concentrations of ciprofloxacin dosed three times

daily are projected to remain in excess of the MPC_{90} and MPC_{50} for 5.5 and 13 hr respectively, of the dosing interval for *P. aeruginosa.* Thus, relevant killing concentrations are also maintained for longer periods for ciprofloxacin. In my preliminary experiments on the killing of *P. aeruginosa* by ciprofloxacin and levofloxacin, MIC concentrations failed to achieve $\geq$ -3 log killing and mutant colonies could be readily recovered from cultures treated at the MIC drug concentration. As expected, when cultures were treated at the MPC drug concentration, significant killing was observed and mutant growth was inhibited. Mutant colonies were killed by ciprofloxacin and levofloxacin MPC drug concentrations within a 2-to-4 hr period, however, the entire contents of the cultures were not sampled and viable mutant colonies may have been missed as a result. Nevertheless, these experiments demonstrate that increased killing is observed at the MPC drug concentration and mutant growth is prevented. Killing of characterized target mutants and detailed analysis of the drug concentrations at the sites of infection will help to further define the length of time ciprofloxacin and levofloxacin MPC concentrations should be targeted in treatment of infection caused by *P. aeruginosa.*

Antibiotic extrusion by efflux pumps is a major determinant of antimicrobial resistance in *P. aeruginosa* and four MDR efflux pumps with broad specificities have been identified in clinical isolates (264, 265, 291). The four major efflux systems which contribute to apparent fluoroquinolone resistance in strains of *P. aeruginosa*, include MexAB-OprM (206, 208, 294), MexCD-OprJ (190, 292), MexEF-OprN (189),

and MexXY-OprM (161, 240, 352). The selection of non-gyrase mutants at low and moderate fluoroquinolone concentrations raised the question of whether activity against efflux mutants of *P. aeruginosa* could be used to account for differences in mutant selection between ciprofloxacin and levofloxacin. However, the relevance of efflux mechanisms in resistance of clinical strains pf *P. aeruginosa* has rarely been demonstrated, mainly because an experimental approach for accurate measurement of the rates of export of antibiotics into bacterial cells is lacking. The selection of efflux mutants at low and moderate quinolone concentrations was demonstrated by Kohler *et al* (190) who were able to detect spontaneously resistant colonies which expressed efflux pumps after incubation when 10^9-to- 10^{10} CFU/ml cultures were challenged with multiple concentrations of the fluoroquinolone MIC. The authors were able to conclude that the preferred resistance mechanism selected by *P. aeruginosa* in response to a single exposure of quinolones at low concentrations (close to the MIC) is antibiotic efflux and not alteration of the gyrase gene (190).

As expected, when mutants over-expressing four efflux systems were tested against increasing fluoroquinolone concentrations, a shift in the recovery of resistant colonies, toward higher drug concentrations, was observed. For all strains tested, a clear distinction between the MIC and the MPC measurements could be observed as colonies continued to form at or above the MIC drug concentration. It is postulated that resistance due to active efflux can be overcome *in vivo* with higher fluoroqouinolone concentrations or more frequent dosing. Thus, in the context of minimizing resistance

in cells expressing MDR efflux pumps, the key factor will be the relative degree that efflux-expressing strains shift the MPC (toward higher drug concentrations) and the mutant selection curve. Direct comparison of the anti-pseudomonal activity for ciprofloxacin and levofloxacin, based on the MPC values, revealed that ciprofloxacin was on average 2.9-fold more active against first-step mutants selected from strains of *P. aeruginosa* which overexpressed one of four different efflux systems. The overall change in susceptibility (based on differences between the MIC and MPC) for ciprofloxacin and levofloxacin was not remarkable for any of the efflux mutants tested, but changes in susceptibility to levofloxacin tended to be less than those for ciprofloxacin. Higher MPC values for levofloxacin resulted in low AUC/MIC ratios which have been linked to an increased potential of a fluoroquinolone to select for resistance (115). MPC results for n*fxB* and *nfxC* mutants of *P. aeruginosa* suggest that mutants selected from strains expressing MexCD-OprJ and MexCD-OprN efflux systems may results in clinical resistance. Sequence analysis of the *gyrA* and *parC* regions in the recovered mutant are now required to determine if mutants selected from *nfxB* mutants represent *gyrA* mutants. Selection of resistant mutants from 10^{10} CFU/ml cultures of *P. aeruginosa* which overespressed one of four different efflux pumps resulted in lower levofloxacin AUC/MPC values when compared to AUC/MPC values obtained for ciprofloxacin. Differences among levofloxacin MPC results for the four efflux mutants tested suggest that activity of levofloxacin against mutants selected from strains expressing different efflux pumps is not equal. Thus, differences in mutant

selection between ciprofloxacin and levofloxacin among strains of *P. aeruginosa* may reside in the preferential ability to restrict the growth of specific efflux mutants of *P. aeruignsoa*. This suggestion is in good agreement with previously reported results by Kohler *et al* (190) who demonstrated that quinolones vary in their capacity to select efflux systems *in vitro*. For older quinolones such as flemequine and nalidixic acid, *nfxC* efflux mutants were selected at higher concentrations and were associated with the highest MICs. In contrast, newer quinolones, which included ciprofloxacin, trovafloxacin and sparfloxacin, selected *nfxB* at 2-to-4 times the MIC and *nfxB* mutants were associated with the highest levels of resistance. A comparison of ciprofloxacin and ofloxacin, the parent drug of levofloxacin (levofloxacin is a 50:50 racemate of the active ingredient in ofloxacin), revealed that at higher concentrations, ciprofloxacin exclusively selected *nfxB* mutants, while ofloxacin selected combinations of *nfxB* and *nfxC* mutants. These results demonstrate the differential selection of efflux systems by quinolones in *P. aeruginosa* and support the hypothesis that differences in mutant selection between ciprofloxacin and levofloxacin are attributed to a differential ability of a fluoroquinolone to restrict the selection of first-step mutants from *P.aeruginosa* strains which express different efflux pumps. This hypothesis is of particular importance in light of recent publications demonstrating the additive effects of multiple MDR efflux pumps on reduced susceptibility in *P. aeruginosa* (208, 219, 296). When I analyzed the recovery of mutants from 10^{10} CFU/ml cultures of efflux mutants of *P. aeruginosa,* selection of resistant mutants was approximately 3-fold higher for

levofloxacin when *nfxB* and *nfxC* efflux mutants were challenged against increasing concentrations of ciprofloxacin and levofloxacin and suggests that the MexCD-OprJ (*nfxC*) efflux may be a more efficient pump for levofloxacin than for ciprofloxacin in isolates of *P. aeruginosa*. This finding is supported by a recent *in vivo* study by Jumbe *et al* (179) who used type-specific efflux pump inhibitors alone and in combination to verify the central role efflux pumps play in the initial emergence of resistance to levofloxacin. With therapy, early time points saw the selection of the MexEF-OprN pump. Later time points demonstrated that the predominant pump system overexpressed in response to challenge with levofloxacin was MexCD-OprJ.

In the context of MPC testing, an additional question remains; do efflux mutants represent first-step resistant cells as defined by the MPC measurement? Efflux expression in *P. aeruginosa* is constitutive and thus, is likely to operate to some degree in susceptible populations. It is possible that cumulative decreases in intracellular drug concentrations for cultures containing $>10^9$ CFU/ml result in increased resistance; an observation not seen with lower numbers of cells (i.e., 10^5 or 10^6 CFU/ml). Thus, in a population of cells 10,000 times greater than those used in conventional susceptibility tests, the cumulative level of efflux expression results in a shift toward higher levels of resistance which can be overcome by higher fluoroquinolone concentrations (i.e., the MPC). In this context, efflux mutants may not represent first-step mutants, as defined by the conventional definition of MPC, however, in the *nalB* multidrug-resistant mutant, overexpression of the mexA-mexB-oprM operon in strains of *P. aeruginosa* has

been associated with a point mutation in *mexR*, the regulator gene of the efflux operon, leading to a predicted substitution of Trp for Arg at position 69 in the encoded peptide, MexR (294). Additional point mutations in the regulatory genes of *nalB, nfxB* and *nfxC* have recently been described (219, 347). Mutations occurring in regulatory efflux genes are also affected by mutational frequencies that are likely to be influenced by population dynamics. Thus, mutational events that contribute to efflux expression may represent first-step mutants. As investigation into MPC expands to include additional antimicrobial agents, other mechanisms of resistance and specific host-drug-pathogen combination, the definition of MPC is likely to expand as well. Although the MPC was initially defined with respect to susceptible populations, the idea applies to any step in the process of gradual accumulation of mutations (85). In a broad sense, the MPC correlates with the MIC of the most-resistant mutant present in the bacterial population. Reductions in intracellular drug concentrations may raise the MPC in strains expressing efflux mechanisms or may then raise the MIC of the target mutants and, thereby, also raise the MPC. The concentration boundaries of the MSW place no restriction on the types of mutants selected (i.e., target versus efflux) and the mechanism of resistance need not be known to use the selection window idea for restricting the development of resistance. Restricting the selection of resistant mutants of *P. aeruginosa* is likely to be a function of both efflux and target mutations. Selection of resistant mutants within strains expressing efflux pumps revealed that levofloxacin was less active against both

first-step resistant mutants (characterized and uncharacterized) and efflux mutants when 10^{10} CFU/ml cultures were tested.

MPC results obtained for 155 clinical isolates of *P. aeruginosa* indicate that no current dosage of ciprofloxacin or levofloxacin is expected to exceed MPC_{90} results for the entire duration of the dosing interval. Thus, minimizing the selection of resistant mutants of *P. aeruginosa* with fluoroquinolone is likely to require combination therapy; however, limited data exists for MPC measurements of other antimicrobial agents alone or in combination. The primary MICs or MPCs are often the criteria utilized by clinicians in selecting appropriate combinations of antimicrobials and, many times, only those antibiotics to which the organism is interpreted as susceptible are used. This practice appears to assume that synergism is obtained only when the organism is susceptible to both antibiotics. Unfortunately, with the increased incidence of MDR *P. aeruginosa* and other pathogens, the clinician is often faced with only one agent, or class of agents, that the pathogen is susceptible to. Combination fluoroquinolone/ceftazidime MPC results restored susceptibility (as determined by a NCCLS breakpoints for susceptibility) in at least one of the agents used in combination for 66% of the ciprofloxacin plus ceftazidime tested isolates and 50% of levofloxacin plus ceftazidime tested isolates. While the remaining strains did not produce combination MPCs that would result in a susceptible designation, all combination results increased the length of time inhibitory concentrations are expected to remain in excess of the MPC. The combination of ciprofloxacin plus ceftazidime extended the

amount of time ciprofloxacin and ceftazidime inhibitory concentrations are maintained for an average of 6.5 and 7.6 hr, respectively, (based on 400 mg T.I.D. ciprofloxacin, 1 g Ceftazidime once daily). Levofloxacin/ceftazidime combinations increased the time levofloxacin and ceftazidime concentrations are in excess of the combination MPC by an average of 1.45 and 3.48 hr, respectively. Additionally, combination MPC results revealed that for every strain tested, at least one of the agents was in excess of the MPC for $\geq$11 hr of a potential 24 hr dosing interval.

The mechanistic difference in anti-pseudomonal activity between ciprofloxacin and levofloxacin used in combination with ceftazidime is still undetermined and may attributed to differences in inherent activity of each compound which become pronounced under combination testing conditions. In an examination of the activity of levofloxacin in combination with ceftazidime, Visalli *et al* (345) reported a 12% synergy between levofloxacin and ceftazidime, which contrasts sharply with the 75% reported by Bustamant *et al* (47) with ciprofloxacin/ceftazidime combinations. These studies highlight the observed differences in the anti-pseudomonal activity of ciprofloxacin and levofloxacin. In my experiments, half of the isolates tested showed no decrease in levofloxacin MPC results obtained in combination. According to the selection window hypotheses, antimicrobial concentrations at the site of infection should be kept outside the widow to avoid selective enrichment of resistant mutants. For fluoroquinolones and *P. aeruginosa*, MPC results suggest that neither ciprofloxacin nor levofloxacin is expected to maintain concentrations above the MPC_{90} for a

significant duration of the dosing interval, so combination therapy is required to effectively lower the MPC and the upper boundary of the MSW.

The exact mechanism(s) of action involved in combination therapy with different drug combinations is not known with certainty and often times "synergy" is used to describe an increase in effect between two agents that could not demonstrate activity independently. Enhanced uptake, mutual killing, macrophage activity and increased permeabilization of the outer membrane have all been suggested as possible mechanisms in the explanation of success with combination therapies (48, 141, 239). Conceptually, a combination treatment regimen containing two or more drugs of different classes should require at least two distinct resistance mutations for the pathogen to grow. For example, if two agents with differing mechanisms of action (which also lack cross-resistance) were to fall within the selection window, organisms would still require two resistance-conferring mutations is order to express resistance. The simultaneous occurrence of two such mutations is expected to occur much less frequently than for a mutational event resulting in a single drug-resistant mutation. This rationale has been used effectively in the combination treatment of *M. tuberculosis*. Consequently, combination therapy with two distinct antibiotic types provides a way to reduce mutant selection using moderate concentrations of compound that may individually have very high MPC values. The role of combination testing and the MPC requires further investigation and many questions remain. For example, combination MPC testing may produce a synergistic result which effectively reduces

the MPC of both antimicrobials such that both agents are expected to remain in excess of the MPC for the duration of the dosing interval. In some cases, such as *M. tuberculosis*, the MPC of the first antimicrobial agent may be outside an achievable drug concentration even when used in combination(s) with other agents, but when combination therapy with multiple agents that have different molecular targets is employed, the ability of the bacteria to develop resistance is reduced. For this situation, matching the PK/PD profiles of multiple agents to ensure that concentrations will rise and fall above and below the MIC simultaneously could be used to help restrict the selection of resistant mutants. If at any time during the dosing interval concentrations of one of the drugs falls below the MIC and the remaining drug is kept above the MIC, then resistant mutants may be selected. In vitro experiments

In vitro experiments have illustrated that first-step resistant mutants are selected by fluoroquinolone concentrations when 10^{10} CFU/ml are exposed to increasing fluoroquinolone concentrations above the MIC of the wild-type strain. Experimentally, the MPC can be measured in a two-stage decline in CFUs when 10^{10} cells are applied to agar containing increasing fluoroquinolone concentrations. Mutant enrichment and subsequent outgrowth was illustrated in killing experiments preformed using 10^{10} CFU/ml cultures tested at the MIC drug concentrations. However, direct *in vivo* evidence confirming the selection of resistant mutants of *P. aeruginosa* with fluoroquinolone therapy in infections containing $>10^9$ cells has not been obtained. I adapted an abscess rat model (306) to test the hypothesis that resistant mutants could be

recovered from animals implanted with 10^{10} CFU/ml cultures of *P. aeruginosa* when given I.V. doses of fluoroquinolones. An isolate of *P. aeruginosa* (CBRH 25138) with intermediate susceptibility to ciprofloxacin and levofloxacin was chosen as the infecting organism since it represented a potentially difficult-to-treat pseudomonal infection. Previous *in vitro* experiments indicated that susceptibility differences between mutants selected from this isolate and parental organisms would be large enough to allow me to monitor mutant selection using 2-fold susceptibility testing. *P. aeruginosa* was recovered from a total of 27 specimens (which included infected tissues, swabs, and blood cultures) and from 9 different animals given doses of either ciprofloxacin of levofloxacin (Table 4.3.10). Nearly 20% of the total number of recovered organisms demonstrated elevated susceptibilities to both ciprofloxacin and levofloxacin, suggesting the selection of resistant organisms. The recovery of *P. aeruginosa* from the infected animals was not a surprising finding and virtually all of the animals tested became bacteremic. Thus, the intent of the experiment was to demonstrate that cultures of *P. aeruginosa* recovered from infected rats represented mutant organisms (with respect to parental susceptibility) which were selected from 10^{10} CFU/ml cultures when treated with fluoroquinolones. Twenty-four percent of the specimens recovered from rats given a once a day dose of ciprofloxacin or levofloxacin showed increases in the MICs to ciprofloxacin and levofloxacin and correlated with an average 2-fold increase in the MIC to ciprofloxacin and 4-fold increase in MIC to levofloxacin. The MIC of the recovered mutants were 4 and 16 μg/ml for ciprofloxacin and levofloxacin,

respectively, and were in agreement with the MPC for the initial strain used to create infection. One specimen showed a 4- and 8-fold increase in the ciprofloxacin and levofloxacin MICs that may relate to the difference in serum versus tissue concentrations of fluoroqouinolones.

These results are based on preliminary experiments using a single isolate of *P. aeruginosa* and relatively small numbers of animals and, as such, require additional testing. However, based on these results, a number of observations can already be made. Resistant organisms were recovered from 3/9 animals tested and phenotypic analysis of resistance was consistent with the hypothesis that first-step resistant mutants are selected from within 10^{10} CFU/ml cultures of *P. aeruginosa* when tested by fluoroquinolones at sub-MPC drug concentrations. This result is consistent with my *in-vitro* experiments. Resistant organisms were recovered from rats after three days of fluoroquinolone treatment, suggesting that mutant organisms pre-exist in populations in excess of 10^7 CFU/ml and can be selected for and enriched by sub-inhibitory drug concentrations. The degree of fluoroquinolone resistance in the recovered mutants was 4-fold higher for levofloxacin than for ciprofloxacin, which is consistent with MPC_{90} results. Mutant organisms were only recovered from animals given a once daily dose of fluoroquinolone. If fluoroquinolone concentrations drop below the MPC, and if the decline of the susceptible populations occurs quickly, either as function of antibiotic action or immune deviation, then mutant enrichment is likely to occur for a majority of the dosing interval giving rise to the selective enrichment of mutant organisms. A

T.I.D. dose, which results in higher concentrations near or above the MPC for a longer period will inhibit first-step resistant mutants and prevent the selection of resistant organisms. For animals give T.I.D. doses of ciprofloxacin or levofloxacin, it is possible that mutant populations were not enriched above threshold that would allow them to detected during standardized susceptibility testing. DNA sequence analysis of *gyrA* and *parC* QRDR regions in the recovered mutants will help to correlate increased fluoroquinolone resistance with potential target mutations. Comparison of target sequences in the mutants recovered from infected rats could then be compared with those obtained under *in vitro* testing to demonstrate that the same mutant variant was selected *in vivo*.

5.0 Comparison of the MIC, MBC, and MPC Measurements for Ciprofloxacin, Levofloxacin and Garenoxacin Against Enteric Gram-negative UTI Pathogens

5.1 Abstract

Acute, uncomplicated UTIs are among the most commonly encountered bacterial infections. Management of these infections has been made more complicated by the trend toward increasing antimicrobial resistance, especially to β–lactams (i.e., ampicillin) and trimethoprim/sulfamethoxazole (TMP-SMX), among uropathogens in the last decade. Fluoroquinolones are suggested as alternative antimicrobials for the treatment of UTIs in communities for which TMP-SXT resistance is $\geq$ 10%. The MPC represents a novel susceptibility parameter designed to minimize the selection of first-step resistant mutants present in large, $\geq 10^{10}$ CFU/ml, cultures. In the following work, I measured MPC results for 80 clinical enteric, Gram-negative urinary isolates and 20 isolates of *P. aeruginosa* against ciprofloxacin, levofloxacin and an experimental fluoroquinolone, garenoxacin. Ciprofloxacin MPC results for *C. freundii, E. cloacae, E. coli, K. pneumoniae,* and *P. aeruginosa* were 0.5, 1, 1, 1 and 4 μg/ml, respectively. MPC results for levofloxacin against *C. freundii, E. cloacae, E. coli,* , *K. pneumoniae* and *P. aeruginosa* were 1, 2, 4, 2 and 16 μg/ml, respectively. MPC results for garenoxacin against *C. freundii, E. cloacae, E. coli, K. pneumoniae,* and *P. aeruginosa* were 1, 8, >8, 4 and $\geq$32 μg/ml, respectively. For ciprofloxacin against *E. cloacae, E. coli*, and *K. pneumoniae* and for levofloxacin against *C. freundii, E. coli,* and *K. pneumoniae,* MPC results were below susceptible breakpoints. Analysis of MPC

results in relation to the PK for garenoxacin, suggests that concentrations are in excess of MPC results for the entire length of the dosing interval for *E. coli*. Overall, the MPC results for ciprofloxacin and levofloxacin were within clinically achievable and sustainable drug concentrations for > 24 hr of the dosing interval against all organisms tested. These results further demonstrate that MPC is a novel susceptibility measurement, reflecting drug activities against first-step resistant organisms present as sub-populations within overall susceptible populations and, as such, is distinct from MIC and MBC measurements of susceptibility. The application of the MPC to fluoroquinolones and the management of UTI represents a situation where high levels of *in vitro* activity, based on low MICs, is reflected in low MPC values for most of the organisms tested. Incorporation of MPC results into current fluoroquinolone dosing in UTIs represents a realistic approach for preventing the further selection of resistant organisms associated with these infections.

5.2 Introduction

UTIs are one of the most commonly infectious diseases for which antibiotic therapy is prescribed (350). In the United States, UTIs account for nearly 8 million physician visits per year and > 100,000 hospital admissions (182, 350). Gram-negative bacteria typically account for a large proportion of UTIs with *E. coli* accounting for 75-to-90% of all UTIs (139, 169, 261). A number of different antimicrobials are used in the treatment of UTI which include (TMP-SMX), amoxicillin, cephalexin, nitrofurantoin and fluoroquinolones such as ciprofloxacin, levofloxacin, and, most recently, gatifloxacin (29, 261, 350). Emerging antimicrobial resistance is beginning to impact on the empiric treatment of UTIs. For example, among *E. coli* isolates, 33-to-60% of the bacterial strains causing cystitis and phylonephritis demonstrate resistance to either amoxicillin or ampicillin (121, 182, 261), and up to 22% of strains may be resistant to TMP-SMX (330). In comparison, resistance to the commonly used fluoroquinolones ciprofloxacin and levofloxacin remains low, at approximately 2-to-5% (182, 184).

Many characteristics of the fluoroquinolones make them ideal agents for the management of UTIs. As a class, the fluoroquinolones are highly active *in vitro* against nearly all significant urinary pathogens (30). They are able to penetrate the prostate well and most are renally excreted, allowing them to concentrate to very high concentrations in the urine (168, 251). Fluoroquinolones generally have a high degree of oral bioavailability, which allows for oral therapies for infections that traditionally

would have been treated with intravenous antibiotics and their long half-lives allow for once or twice daily dosing. Consequently, fluoroquinolones have been and will continue to be used extensively in the treatment and prevention of UTIs, particularly in situations were TMP-SMX resistance is $\geq$ 10% (350).

When fluoroquinolones were first introduced in the 1980s, nearly all organisms recovered from patients with UTIs were considered susceptible *in vitro*. Even today, fluoroquinolones remain among the most active oral agents against urinary tract isolates (26, 101). However, over the past decade a number of studies have reported a substantial increase in the prevalence of fluoroquinolone resistance among urinary tract isolates and the prevalence of fluoroquinolone resistance has progressively risen. For example, fluoroquinolone resistance levels among urinary isolates of *E. coli* have been reported as high as 25% within Latin America (121). More disturbingly, many isolates commonly now have single point mutations in *gyrA* that presage progression to full resistance with additional mutations. Therefore, minimizing the step-wise selection of fluoroquinolone-resistant Gram-negative bacteria is an important factor in preventing further increases in fluoroquinolone resistance among Gram-negative uropathogens. The MPC is a novel susceptibility measurement designed to identify and inhibit the most-resistant first-step resistant organisms present in large ($\geq 10^{10}$ CFU/ml). heterogeneous bacterial cultures. In the following work, I measured MIC, MBC and MPC results for 100 Gram-negative urinary tract isolates for two commonly prescribed fluoroquinolones, ciprofloxacin and levofloxacin, and for a developmental

fluoroquinolone garenoxacin (BMS 284756) which has been shown to possess activity against Gram-negative UTI pathogens *in vitro* (59, 118).

5.3 RESULTS

Clinical isolates of *C. freundii* (n=20), *E. cloacae* (n=20), *E. coli* (n=20), *K. pneumoniae* (n=20) and *P. aeruginosa* (n=20) obtained from urinary tract specimens were tested by MIC, MBC and MPC as described in the Materials and Methods section. Comparison of MPC_{90} values revealed a relative hierarchy in fluoroquinolone potency (table 5.3.1).

Against the 20 *C. freundii* isolates, modal MIC values were ≤0.06, 0.25 and ≤0.06-0.125 μg/ml, respectively, for ciprofloxacin, levofloxacin and garenoxacin, while modal MPC values were 0.125-0.5, 0.5 and 0.25 μg/ml, respectively. For the 20 *E.coli* isolates (Figure 5.3.1) the modal MIC values for ciprofloxacin, levofloxacin and garenoxacin was ≤0.06, while the modal MPC values were 0.125-0.25, 0.25 and 0.25-0.5 μg/ml, respectively. Ciprofloxacin and levofloxacin had the same modal MIC values against strains of *E. cloacae* (≤0.06 μg/ml), whereas the values for garenoxacin were between ≤0.06-0.125 μg/ml. The modal MPC values against *E. cloacae* strains were 0.125 μg/ml for ciprofloxacin and 1 μg/ml for levofloxacin and garenoxacin. Against the 20 clinical strains of *K. pneumoniae*, modal MIC values were ≤0.06 μg/ml for ciprofloxacin, ≤0.06-0.125 μg/ml for levofloxacin and 0.125 μg/ml for garenoxacin; modal MPC values were 0.5 μg/ml, 1 μg/ml and 1-4 μg/ml, respectively. Finally, the modal MIC values against the 20 clinical isolates of *P. aeruginosa* were ≤0.06 mg/ml,

Table 5.3.1 MIC, MBC, and MPC Distributions for UTI Isolates Against Ciprofloxacin, Levofloxacin, and Garenoxacin

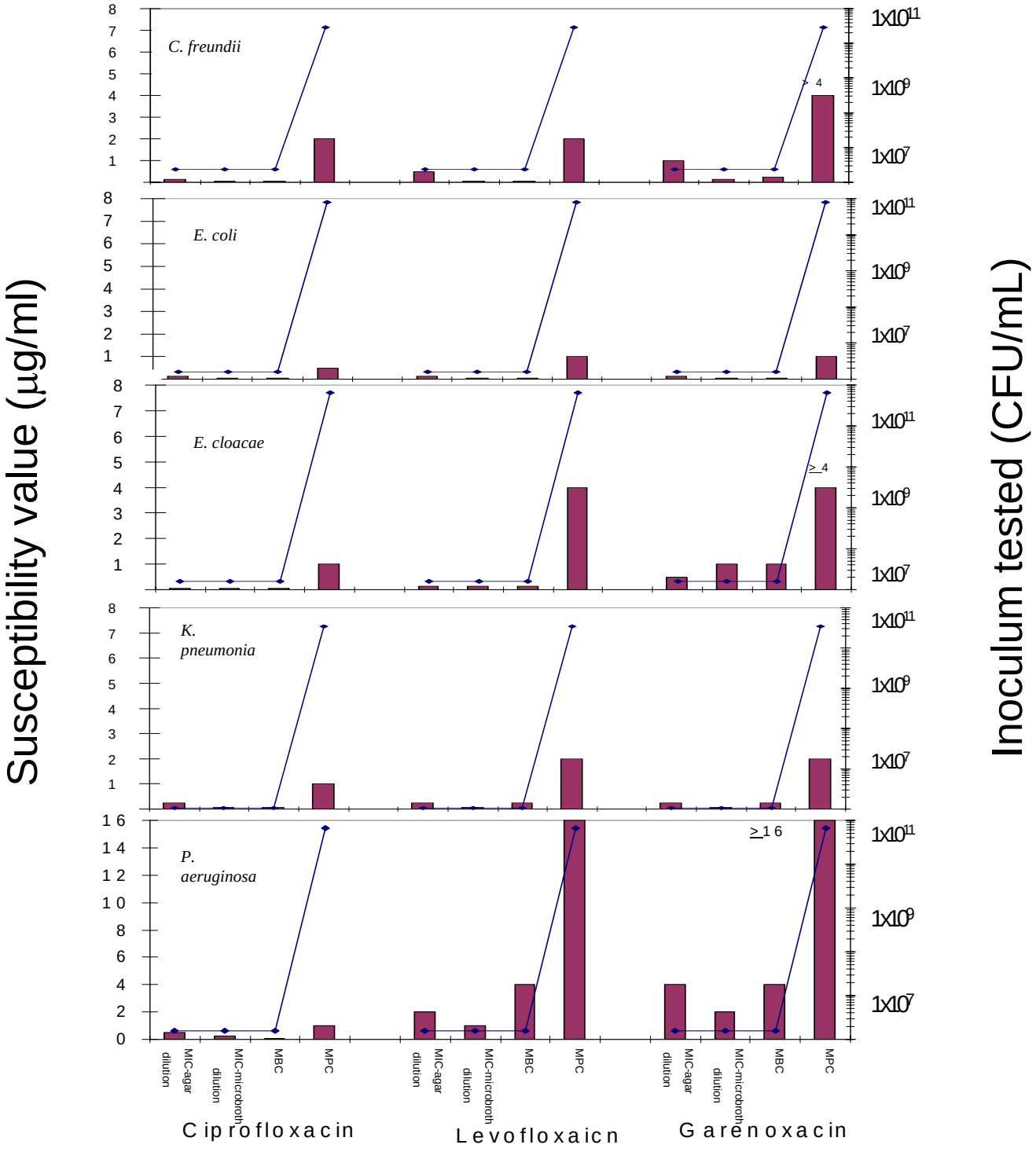
Pathogen	FQ	Measurement	Fluoroquinolone Susceptibility μg/ml										Mean number of cells tested (CFU/ml)	MPC/susceptibililty measurement	Result for 90% of isolates
			≤0.06	0.125	0.25	0.5	1	2	4	8	16	>32			
C. freundii (n=20)	Cpx	MIC-AD	14	4	1	1	0	0	0	0			$2.33x10^6$	16	0.125
		MIC-MB	16	2	0	2	1	0	0	0			$2.33x10^6$	4	0.5
		MBC	15	1	0	1	1	0	0	0			$2.33x10^6$	4	0.5
		MPC	0	5	3	5	3	4	0	0			$2.87x10^{10}$	----	2
	Grx	MIC-AD	5	5	2	0	3	1	4	0			$2.33x10^6$	2	4
		MIC-MB	3	5	3	1	2	2	3	1			$2.33x10^6$	2	4
		MBC	3	5	3	1	2	2	3	1			$2.33x10^6$	2	4
		MPC	0	0	4	2	4	3	4	4			$2.87x10^{10}$	----	8
	Lfx	MIC-AD	5	5	6	4	0	0	0	0			$2.33x10^6$	4	0.5
		MIC-MB	9	3	5	3	0	0	0	0			$2.33x10^6$	4	0.5
		MBC	5	5	5	3	2	0	0	0			$2.33x10^6$	1	1
		MPC	0	1	0	8	3	8	0	0			$2.87x10^{10}$	----	2
E. cloaceae (n=20)	Cpx	MIC-AD	18	1	0	1	0	0	0	0			$1.60x10^6$	16.6	≤0.06
		MIC-MB	18	0	0	1	1	0	0	0			$1.60x10^6$	16.6	≤0.06
		MBC	18	0	0	1	1	0	0	0			$1.60x10^6$	16.6	≤0.06
		MPC	4	7	5	1	1	1	1	0			$6.62x10^{10}$	----	1
	Grx	MIC-AD	7	7	3	2	1	0	0	0			$1.60x10^6$	>16	0.5
		MIC-MB	7	7	2	0	4	0	0	0			$1.60x10^6$	>8	1
		MBC	7	7	2	0	4	0	0	0			$1.60x10^6$	>8	1
		MPC	0	0	0	4	8	3	1	>4			$6.62x10^{10}$	----	>8

	Lfx	MIC-AD MIC-MB MBC MPC	14 15 15 0	3 3 3 1	2 1 1 3	1 0 0 6	0 1 1 7	0 0 0 0	0 0 0 2	0 0 0 1			$1.60x10^{6}$ $1.60x10^{6}$ $1.60x10^{6}$ $6.62x10^{10}$	4 32 32 ----	0.25 0.25 0.25 4
E.coli (n=20)	Cpx	MIC-AD MIC-MB MBC MPC	17 19 19 1	2 1 1 8	1 0 0 8	0 0 0 3	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0			$1.63x10^{6}$ $1.63x10^{6}$ $1.63x10^{6}$ $7.78x10^{10}$	4 8.3 8.3 ----	0.125 ≤0.06 ≤0.06 0.5
	Grx	MIC-AD MIC-MB MBC MPC	15 19 19 0	4 1 1 0	0 0 0 8	1 0 0 8	0 0 0 3	0 0 0 1	0 0 0 0	0 0 0 0			$1.63x10^{6}$ $1.63x10^{6}$ $1.63x10^{6}$ $7.78x10^{10}$	8 16.6 16.6 ----	0. 125 ≤0.06 ≤0.06 1
	Lfx	MIC-AD MIC-MB MBC MPC	16 19 19 0	2 1 1 0	1 0 0 12	1 0 0 5	0 0 0 2	0 0 0 1	0 0 0 0	0 0 0 0			$1.63x10^{6}$ $1.63x10^{6}$ $1.63x10^{6}$ $7.78x10^{10}$	4 16.6 16.6 ----	0. 25 ≤0.06 ≤0.06 1
K. pneumoniae (n=20)	Cpx	MIC-AD MIC-MB MBC MPC	14 19 18 1	2 0 1 3	3 1 0 7	1 0 1 3	0 0 0 4	0 0 0 2					$1.06x10^{6}$ $1.06x10^{6}$ $1.06x10^{6}$ $3.42x10^{10}$	4 16 16 ----	0.25 ≤0.06 ≤0.06 1
	Grx	MIC-AD MIC-MB MBC MPC	0 4 3 0	10 12 12 0	8 4 5 0	2 0 0 2	0 0 0 6	0 0 0 6	0 0 0 6				$1.06x10^{6}$ $1.06x10^{6}$ $1.06x10^{6}$ $3.42x10^{10}$	16 16 16 ----	0.25 0.25 0.25 4
	Lfx	MIC-AD MIC-MB MBC MPC	9 10 7 0	9 5 8 0	0 3 1 1	2 3 1 4	2 1 2 10	0 1 1 4	0 0 0 1				$1.06x10^{6}$ $1.06x10^{6}$ $1.06x10^{6}$ $3.42x10^{10}$	2 2 2 ----	0.5 1 1 1

P. aeruginosa (n=20)	Cpx	MIC-AD	9	0	7	2	2	0	0	0			$1.60x10^6$	4	1
		MIC-MB	2	6	8	3	2	1	0	0			$1.60x10^6$	4	1
		MBC	2	4	7	1	4	2	0	0			$1.60x10^6$	2	2
		MPC	0	0	0	0	0	2	7	2			$6.6x10^{10}$	----	4
	Grx	MIC-AD			1	6	5	5	2	0	1	0	$1.60x10^6$	$\geq$8	4
		MIC-MB			0	0	4	9	5	1	0	1	$1.60x10^6$	$\geq$4	4
		MBC			0	0	1	4	10	4	0	1	$1.60x10^6$	$\geq$4	8
		MPC			0	0	0	0	0	2	10	8	$6.62x10^1$	----	$\geq$32
	Lfx	MIC-AD			4	7	5	3	1	0	0	0	$1.60x10^6$	8	2
		MIC-MB			3	6	7	1	3	0	0	0	$1.60x10^6$	4	4
		MBC			1	5	4	7	3	0	0	0	$1.60x10^6$	4	4
		MPC			0	0	0	0	4	10	5	1	$6.62x10^{10}$	----	16

Cpx. Ciprofloxacin. Lfx. Levofloxacin. Grx. Garenoxacin. MIC-AD. MIC obtained with agar dilution. MIC-MB. MIC obtained with microbroth dilution.

Figure 5.3.1 MIC, MBC and MPC Results for 100 Gram-negative Urinary Isolates of *C. freundii, E. coli, E. cloacae, K. pneumoniae* and *P aeruginosa* for Ciprofloxacin, Garenoxacin, and Levofloxacin



MIC, MBC and MPC distributions for *C. freundii* (n=20), *E. coli* (n=20), *E. cloacae* (n=20), *K. pneumoniae* (n=20), and *P. aeruginosa* (n=20) against ciprofloxaicn, levofloxaicn, and garenoxacin. MIC-agar refers to MICs performed using the agar dilution method, MIC-microbroth refers to performed using the microbroth dilution method. Lines indicate the number of cells tested for each test.

0.5 μg/ml and 1-2 μg/ml, respectively for ciprofloxacin, levofloxacin and garenoxacin. The modal MPC values were 4 μg/ml, 8 μg/ml and 16 μg/ml, respectively. MPC distributions did not directly correlate with MIC results. For example, all agents tested had MIC_{90} values of $\leq$0.06 μg/ml against *E. coli* but ciprofloxacin was 2-fold more active by MPC than both levofloxacin and garenoxacin. Based on MIC values, ciprofloxacin was at least 8-fold more active than levofloxacin against isolates of *K. pneumoniae,* but both ciprofloxacin and levofloxacin reported MPC_{90} values of 1 μg/ml. Garenoxacin was 4-fold more active than levofloxacin against *K. pneumoniae* as determined by the MIC_{90}, but was 2-fold less active by MPC. Low MPC results tended to correlate with low MIC results, however, MPC measurements against the Gram-negative organisms tested could not be directly extrapolated from MIC results in all instances, which were reflected in the ratios of MPC/MIC. Although all three quinolones demonstrated high levels of *in vitro* activity against strains of *E. coli*, the range between the MIC and the MPC was among the highest observed for any pathogen. The MPC results obtained for *C. freundii*, *E. cloacae, E. coli*, *K. pneumoniae* and *P. aeruginosa* for the three quinolones were generally 2-4, 8-16, 16, 16 and 4 times greater than the MIC. The only exception to this trend was found for levofloxacin against *K. pneumoniae*, were a 2-fold difference between the MIC and MPC was observed. As a greater number of organisms are tested, the MPC/MIC ratio may serve as a general guideline for assessing quinolone MPCs against different

pathogens through batch studies that may eliminate the need for determining an individual MPC.

The lowest MPC results were observed for isolates of *E. coli*, followed by *K. pneumoniae, E. cloacae, C. freundii* and *P. aeruginosa* (Table 5.3.1). MPC results for ciprofloxacin and levofloxacin against Gram-negative urinary tract pathogens were generally low. For ciprofloxacin, the MPC results for *E. cloacae, E. coli,* and *K. pneumoniae* were all at or below the susceptible breakpoint as set by the NCCLS ($\leq$ 1 μg/ml). The MPC results for *C. freundii* and *P. aeruginosa* were at an intermediate (2 μg/ml) and above a resistant (4 μg/ml) breakpoint. For levofloxacin, MPCs against *C. freundii, E. coli,* and *K. pneumoniae* were below a susceptible breakpoint ($\leq$2 μg/ml), while MPCs against *E. cloacae* and *P. aeruginosa* were at intermediate (4 μg/ml) and resistant (8 μg/ml) breakpoints. No breakpoints for garenoxacin have been developed, however, based on the published PK (120) and reports on the *in vitro* activity (118), it is hypothesized that only *E. coli* may have low enough garenoxacin MPC values to still be considered susceptible.

5.4 DISCUSSION

Acute, uncomplicated UTIs are among the most commonly encountered bacterial infections. Management of these infections has been made more complicated by the trend toward increasing antimicrobial resistance, especially to β–lactams and TMP-SMX, among uropathogens in the last decade (168). The current standard of therapy for empiric treatment of acute, uncomplicated UTIs is TMP-SXT (350), but the prevalence of resistance to TMP-SMX among uropathogens is increasing (168, 169) and empiric therapy without the guide of laboratory results can no longer ensure success. Consequently, quinolones are increasingly being used as first line agents for the treatment of UTIs and *in vitro* susceptibility results have become important in the management of these infections (26). Laboratory susceptibility results are a valuable tool for clinicians and can be used to guide therapeutic decisions. MICs obtained via microbroth dilution, agar dilution, disk diffusion, or E-tests are typical susceptibility tests preformed in clinical settings (258). The MBC is less commonly preformed and represents concentrations which kill 99% of the culture. The bactericidal action of quinolones generally results in a very tight dispersion between MIC and MBC results, usually ≤1 doubling dilution (247, 270, 346)**.** To date, no studies have specifically addressed the relationship between MBC and MPC measurements. Differences of 2- to 16-fold were noted between MPC and MBC measurements for ciprofloxacin, garenoxacin, and levofloxacin against isolates of *C. freundii, E. cloacae, E. coli, , K. pneumoniae,* and *P. aeruginosa*. MPC results measured for isolates of *C. freundii, E.*

cloacae, E. coli, K. pneumoniae, and *P. aeruginosa* fell within a susceptible NCCLS designation for many of the quinolones tested, however, differences between the MIC/MBC and MPC were observed for every drug-pathogen combination which ranged from 4-to-16-fold for ciprofloxacin, 2-to 16-fold for levofloxacin and 2-to 16-fold for garenoxacin. With the exception of MBC measurements, all susceptibility tests were preformed using identical test volumes, differing only in the number of cells tested. Thus, the MPC measurement is fundamentally different from standardized susceptibility measurements because it utilizes an inoculum that accounts for the presence of first-step resistant cells. Inhibiting the growth of the most-resistant mutant present in subpopulations within susceptible 10^{10} CFU/ml cultures will minimize the step-wise development of fluoroquinolone resistance. No differences in ciprofloxacin and levofloxacin MPC results were seen for *C. freundii, E. coli,* and *K. pneumoniae*. Ciprofloxaicn MPC results were 4-fold lower seen with levofloxacin against isolates of *E. cloacae* and *P. aeruginosa.*

PK/PD analysis of ciprofloxacin and levofloxacin concentrations in healthy volunteers have demonstrated urinary Cmax of 43 and 84 μg/ml, respectively (45, 252). Therefore, ciprofloxacin and levofloxacin concentrations are expected to remain in excess of the MPC for greater than 24 hr of the dosing interval for all Gram-negative uropathogens tested. No published experiments regarding urine C_{max} values for garenoxacin have been determined, but it is expected that urinary C_{max} concentrations will exceed published serum levels. Thus, garenoxacin may be effective at preventing

the selection of resistant organisms of *E. coli* and *K. pneumoniae* if concentrations above the MPC for these organisms can be achieved and maintained.

Fluoroquinolone treatment for UTIs can be used as a model to examine the clinical utility of the MPC measurement. Despite nearly 25 years of documented use of fluoroquinolones in the treatment of UTI, the incidence of fluoroquinolone resistance remains low (112), ranging from 2-to-5% in Canada and the United States (139, 181, 182). Correlations between antimicrobial consumption and resistance for other antimicrobial classes and examination of fluoroquinolone resistance for Gram-negative pathogens outside the urinary tract, implies that a higher incidence of quinolone resistance should be expected among urinary tract pathogens. A possible explanation for the low incidence of fluoroquinolone resistance among Gram-negative urinary tract pathogens is that urinary concentrations of fluoroquinolones may be 100- to 1000-fold higher than those obtained at other anatomical sites (251, 252). Thus, urinary quinolone concentrations are expected to remain in excess of the MPC for a number of Gram-negative pathogens throughout the majority of the dosing interval. This argument does not preclude resistance from occurring; clearly fluoroquinolone resistance rates among Gram-negative pathogens have been described (121). It is possible, particularly within Latin American and developing countries, that quinolone resistance is higher because of the use of less active quinolones such as nalidixic acid and or the use of low dosages which may select resistant isolates (121). High-level resistant organisms may be selected at different sites of infection where MPC values cannot be achieved or

maintained for sufficient periods to prevent the selection of resistant organisms that can then infect the urinary tract. However, high concentrations of quinolones obtained in the urine do not guarantee success as is illustrated by the observation that *in vitro* resistance to TMP-SMX predicts an approximate 50% failure rate with TMP-SMX therapy despite this agent's high urine concentrations and long half life (139, 231, 234, 299, 335, 359). Thus, clinical failures can be expected with fluoroquinolones as well. Sheng *et al* (325) examined sets of isolates from the mid to late 1980s and mid to late 1990s to examine fluoroquinolone resistance in clinically important Gram-negative bacteria in Japan. *E. coli* isolates were highly susceptible to fluoroquinolones prior to 1996; however, by 1996, 20% of the strains had become resistant to one of the fluoroquinolone tested, although ciprofloxacin resistance remained low. The authors also noted a 13-to-20% decline in susceptibility to fluoroquinolones for *Enterobacter* spp. and *P. aeruginosa.* While resistance correlates with the wide spread use of these antimicrobial agents in Japan, increased use alone could not account for these observations. Some strains of *S. marcescens* and *P. aeruginosa* demonstrated *in vitro* resistance prior to the introduction of fluoroquinolones in Japan (229) and many strains demonstrated clonal diversity suggesting that resistance was due to more than one resistant clone. It was further noted that 62% of the patients infected with a fluoroquinolone-resistant bacteria had no previous exposure to the drug (229). Fluoroquinolone-pathogen combinations that result in small MPC/MIC ratios and overall low MPCs (below a breakpoint for resistance) should be targeted in strategies

designed to maximize clinical outcomes while minimizing resistance. Fluoroquinolone use in UTIs for the treatment of infections caused by Gram-negative bacteria describes situations where the initial degree of resistance (based on the MIC) and the potential to selectively amplify resistant subpopulations (based on the MPC) are also low.

The future role of fluoroquinolones in UTI remains strong and the clinical longevity of these agents will be influenced by how resistance evolves. For fluoroquinolones and Gram-negative urinary tract pathogens, the distinction between the MIC and the MPC remains relatively small and concentrations of quinolones in the urine suggest that MPC drug concentrations can be maintained throughout dosing. Therefore, the recognition of spontaneous first-step mutants present within large (i.e., $>10^7$ CFU/ml) susceptible cultures is an important factor in preventing the step-wise selection of quinolone-resistant strains. MPC results for *C. freundii, E. coli and K. pneumoniae* fall beneath a susceptible breakpoint and demonstrate how the MPC measurement can be easily incorporating in current dosing regimes. Urinary concentrations of fluoroquinolones suggest that in organisms with high MPCs such as *E. cloacae* and *P. aeruginosa*, minimizing the selection of resistant mutants may still be accomplished due to the high urinary concentrations achieved and maintained throughout current dosing.

6.0 CONCLUDING REMARKS

Since the introduction of sulfa drugs and penicillin in the 1930s and 1940s, science and medicine have witnessed more than half a century of development and use of antibiotics which have clearly altered the course of medical history and will continue to define the antibiotic era. Past policies for dealing with resistance have at best been only partially effective and despite developments, we continue to face the same challenges encountered at the beginning of the antibiotic era. As an example, in 1960, Gould stated, "we are as yet at an elementary stage in correlating the clinical administrations of antibiotics with *in vitro* sensitivity determinations" (136).

Based on current knowledge, one can surmise three inherent principals involved with antibiotics and resistance:

1. antibiotic resistance is an undeniable fact and will continue to be a problem as long as we use (and misuse) antimicrobial agents,
2. bacteria are remarkably adaptive and will continue to evolve and acquire new mechanisms of resistance to antimicrobial agents, and
3. prior strategies for dealing with antibiotic resistance have failed to slow the progression and in some cases have clearly failed. Therefore, novel approaches for dealing with resistance are required.

In this thesis, I have discussed a novel measure of drug potency that is based on a susceptibility parameter termed the MPC and highlighted how incorporation of the

MPC into the concept of the MSW may be used in an attempt to preserve antimicrobial efficacy, minimize resistance, and maximize clinical outcome.

Experimentally, the MPC can be described in a characteristic two-stage decline in bacterial growth. The first stage of colony decline occurs at fluoroquinolone concentrations which approximate the MIC of the wild-type strain. At fluoroquinolone concentrations above the MIC of the wild-type strain, a plateau in cell death occurs which corresponds to the emergence of drug-resistant mutants. Increasing fluoroquinolone concentrations results in a second decline in colony recovery, leading to the eventual MPC for the given isolate. For any given fluoroquinolone-pathogen combination, the selection of resistant mutants and the corresponding MSW will be different. For example, for organisms which remain highly susceptible to fluoroquinolones, such as *S. pneumoniae*, the plateau in cell death will be relatively short, and the MPC may fall within a susceptible breakpoint for the drug. Conversely, *P. aeruginosa* represents an organism where the differences between MIC and MPC measurements are large and mutant selection occurs over broad concentration ranges. However, despite these differences, the potential utility of the MPC measurement for restricting the selection of fluoroquinolone resistant mutants remain the same.

In chapter 3, I described how the MPC measurement could be used to identify key differences in the anti-pneumococcal activity of six fluoroquinolones deemed clinically equivalent. When applied to drug PK/PD profiles, MPC measurements were used to predict the potential of each agent to select for resistance. In order of

descending activity, a hierarchy of potency based on the ability to inhibit first-step resistant mutants was determined: gemifloxacin>moxifloxacin>gatifloxacin = trovafloxacin >grepafloxacin > levofloxacin. Gemifloxacin, and moxifloxacin were the only compounds tested whose serum/tissue concentrations are expected to remain in excess of the MPC for >12 hr of the dosing interval, suggesting that they may be appropriate for once daily dosing. Levofloxacin selected mutants at the highest concentration of any quinolone tested and serum concentrations of levofloxacin were projected to remain in excess of the MPC for approximately 3 hr of the dosing interval (the lowest of any quinolone tested). Thus, a higher (possibly 750 mg) dose of levofloxacin and/or more frequent dosing may be required to prevent the selection of fluoroquinolone-resistant *S. pneumoniae*, which is consistent with recent reports of levofloxacin-associated clinical failures in the treatment of *S. pneumoniae* (7, 70, 340, 351). Sequence analysis of the QRDR for the *parC* and *gyrA* genes of selected clinical isolates revealed the presence of target mutants which raises concerns about accumulation of fluoroquinolone resistance alleles among clinical isolates of *S. pneumoniae* that are not detected in traditional susceptibility testing procedures. Fluoroquinolone killing of *S. pneumoniae* at the MPC drug concentration resulted in increased killing and bacterial eradication by 24 hr, indicating that targeting the MPC may also have an impact on the speed of clinical resolution. There is little doubt that fluoroquinolone resistance in strains of *S. pneumoniae* has increased during the past decade (53). For example, a number of studies have shown that once the prevalence of

resistance begins to increase noticeably, it can advance from below 5% to above 20% within a few years (13, 175) .

Currently, fluoroquinolone resistance among clinical isolates of *S. pneumoniae* remains relatively low and new strategies/susceptibility testing procedures, such as the MSW and the MPC, can impact the degree of resistance and the rate at which it develops. Past lessons, such as the escalation of penicillin resistance within *S. pneumoniae* have shown us that low dose therapy caused an increase in the carriage of resistant isolates and, after mutant spread to fresh hosts, curing infections required higher doses of penicillin or new derivatives having greater potency or different binding targets. Prolonged and gradual step-wise selection with β-lactams coupled with dissemination of plasmid-borne factors resulted in the selective enrichment of resistant isolates which has made penicillin ineffective against a third of the *S. pneumoniae* isolates in some areas (79, 160). Therefore, MPC testing for fluoroquinolones and *S. pneumoniae* represents a realistic approach for dealing with resistance before it becomes highly disseminated among clinical isolates and is of particular importance in light of the fact that fluoroquinolone treatment for *S. pneumoniae* is often administered in culture-negative patients.

The *in vitro* susceptibility of *P. aeruginosa* to ciprofloxacin and levofloxacin has not always correlated with clinical outcomes and a number of clinical observations suggest that differences in anti-pseudomonal activity are not reflected by the MIC. When clinical isolates of *P. aeruginosa* were tested by the MPC approach, ciprofloxacin

was 2 -to 4-fold more active than levofloxacin. MPC_{90} results indicate that no current dose of levofloxacin is able to exceed the levofloxacin MPC_{90} result for any length of a dosing period. In contrast, a 400 mg I.V. T.I.D. dose of ciprofloxacin is expected to exceed the ciprofloxacin MPC_{90} result for approximately 5.5 hr of the 24-hr dosing interval. Based on these results, I conclude that: i) neither compound should be used in monotherpy, ii) levofloxacin selects and enriches more first step mutants at higher concentrations and, iii) for a longer duration of the dosing interval. Thus, observed differences in clinical outcomes may be attributed to the fact that the levofloxacin MPC is not achieved or maintained under current dosing regimes, allowing first-step resistant mutants to proliferate during therapy. Sequence analysis of regions of the *gyrA* and *parC* genes revealed target mutations in recovered mutants that were not present in parental isolates. Differences in the anti-mutant activity between ciprofloxacin and levofloxacin may reside in the differential selection of resistant mutants. Sequence analysis of the target genes in recovered mutants revealed that levofloxacin may select different target mutants and mutant-selection curves preformed on efflux mutants revealed that levofloxacin was more efficient at selecting efflux mutants at low and moderate concentrations. MPC-based killing experiments revealed that killing at the MPC drug concentration severely restricted mutant growth when the MPC and MIC drug concentrations were directly compared. Preliminary *in vivo* experiments preformed with a rat abscess model of infection revealed that resistant mutants are selected during therapy.

Examination of MPC results for Gram-negative urinary tract pathogens suggested that the MPC measurement could be practically applied in current dosing to prevent the selection of resistant mutants and maximizing clinical outcomes. The MPC measurement and concept of MSW provides a rationale strategy designed to prevent the selection and amplification of *de novo* resistance, however, a number of limitations in MPC testing currently exist. As indicated by Allen (6), the most important difficulty encountered in determination of the MPC lies in achieving the targeted inoculums. Bacterial organisms which do not readily achieve $\geq 10^{10}$ CFU/ml may require additional numbers of MPC plates, for each concentration tested, to ensure that 10^{10} CFU/ml are tested. This procedure does not lend itself to a high throughput clinical setting. If the MPC is to gain utility in a typical clinical setting, streamlining of the methodology may be necessary (6). The application of MPC testing to the clinical microbiology laboratory may require that batch studies be performed on a representative number of bacterial species which could serve as an indicator of activity for the entire species population. If a relationship between MIC and MPC results can be deciphered, then clinicians, microbiologist, pharmacologists and scientists may be able to extrapolate MPC activity directly from the MIC. Advances in molecular diagnostics may allow for the real-time detection of a single first-step mutant within a background of 10^8 cells (215-217). Currently, MPC testing relies on an agar dilution method and some drug-pathogen combinations require broth dilution testing as per NCCLS recommendations.

The application of MPC testing to non-quinolone antimicrobials is an area that requires further investigation. While the majority of experiments describing the MPC have been conducted with fluoroquinolones, additional studies on other antimicrobial agents have been performed (147, 221). Zhoa *et al* (367) published MPC data for chloramphenicol, penicillin G, rifampicin and tobramycin against *E. coli* . Recently, my colleagues and I described the effect of antimicrobial concentration on colony-forming ability of resistant mutant subpopulations of *Mycobacterium smegmatis* and *S. aureus* for chloramphenicol, erythromycin, moxifloxacin, penicillin and tetracycline (221). Ongoing investigations of MPC measurements with macrolides and *S. aureus* and *S. pneumoniae* suggest that MPC studies are relevant to this class of agents (32, 235) Azithromycin-susceptible strains (MICs $\leq$0.5 μg/ml) were tested by MPC. For some isolates, MPC values ranged from 16-$\geq$512 μg/ml and were positive for the *ermC* gene, whereas the wild-type parental strains were negative (Blondeau and Metzler, unpublished observations). The *ermC*-positive isolates also had elevated MIC/MPC values to other macrolide compounds (32). MPC is most easily considered with organisms in which resistance arises as a function of single point mutation which include fluoroquinolones and third generation cephalosporins among others. However, in the broadest sense, resistance to an antimicrobial agent means that a particular microorganism is able to reproduce in the presence of the agent under specified conditions. Resistance may be associated with a specific heritable alteration or induction of protective genes such as those encoding β-lactamses. Heritability would

seem to make resistance an absolute term since changes in DNA primary structure are unequivocal. However, in some cases resistance genes are not fully protective and some drugs, perhaps used at higher concentrations, prove effective. Consequently, the term resistance must be qualified to take into account the antimicrobial drug concentration (85). As initially proposed in the discussion of efflux resistance in Chapter 5, the MSW hypothesis (for which the MPC is the upper boundary) places no restriction on the type of resistance mechanism selected. As bacterial inoculums increase, so too does the heterogeneity of the bacterial culture. Increased inoculums may dilute intracellular targets or enzymatic activity of resistance mechanisms which serve to increase resistance beyond what is measured by the MIC. If under these conditions, higher concentrations of antimicrobial are required to inhibit cultures, then a MPC measurement may apply. In a recent clarification of the mutant selection window, Zhao (366) suggests that once a small fraction of mutants is present in an infected individual or heterogeneous culture, the key idea in preventing the selection of resistance will be whether resistant mutants will be enriched; not how they came into being. In this respect, the MPC may apply to a number of different antimicrobial agents.

The issue of "collateral damage", or the unwanted selection of resistant organisms due to high quinolone concentrations, was an issue raised by Livermore (218) in regard to the MPC. Prolonged low-dose therapy has unquestionably led to the development of resistance, however, dosing based on MPC measurements champions

higher doses administered over a potentially shorter duration of therapy; a hypothesis supported by preliminary killing experiments. The implication that quinolones may directly contribute to the selection of resistant mutants can be inferred based on evidence that quinolones are strong inducers of the S.O.S. response (284-286). However, a number of observations suggest that quinolones contribute minimally to resistance. For example, maximal induction of the S.O.S. response by fluoroquinolones in various organisms typically occurs at concentrations 10-to 15-fold above the MIC values for a number of bacterial species (284). The MPC results, for most bacterial species, are generally 2- to 8-fold greater than typical MIC results. Mutational studies on a LexA$^-$ strain of *E. coli* revealed no differences in the mutational frequency at which quinolone resistance developed when compared to LexA isogenic strains (Xillin Zhao, personal communications).

Perhaps the most important question surrounding the MPC concerns its potential clinical impact. Although *in vitro* observations and retrospective clinical observations support the role of MPC in minimizing the selection of resistant mutants, no direct evidence from animal or human trials are currently available to test the hypothesis that incorporation of MPC-based testing in antimicrobial management will correlate with decreased clinical resistance and improved therapeutic outcomes. In my animal studies, organisms displaying elevated resistance could be recovered from infected animals when a strain with an intermediate MIC was used to create infection. The enrichment of mutant subpopulations *in vivo* is a reasonable explanation for the

recovery of such resistant organisms. A recent report which used a rabbit model of pneumococcal infection to investigate the selection of resistant mutants of *S. pneumoniae* revealed that, after levofloxacin or moxifloxacin treatments, mutants could be recovered from strains with a pre-existing *parC* mutation (99). The authors rationalized this finding by suggesting that strains with a pre-existing *parC* mutation caused drug concentrations to fall below the MPCs of these strains. Further *in vivo* (animal and human trials) are now required to test theimplicatons of the MPC measurement and the MSW concept.

Conventional wisdom suggests that resistant mutants are inherently less "fit" than wild-type cells and as a result, elicit reduced growth rates (129). However, recent data suggests that mutations in *parC* and *gyrA* genes may, on some occasions, not be associated with a physiological deficit (129). Furthermore, in some cases resistance may associated with an increase in fitness, as assessed by increased growth rate (42). The clinical consequence of mutant subpopulations is currently unknown. The theory behind the MPC measurement implies that one resistant mutant is as etiologically important as 100,000 mutants; however, from a clinical perspective, this argument may not hold true. The dissemination of penicillin-resistant *S. pneumoniae* serotypes 3, 14, 19F and 23F demonstrate how the spread of individual resistant clones can impact on global resistance and demonstrates the necessity for minimizing resistance. In the context of *S. pneumoniae,* recent evidence (7, 70) indicates that treatment failures are associated with resistant organisms which were not present at the start of therapy. In

light of these observations, strategies designed to minimize the impact of resistance should incorporate ideas which make it difficult for organisms to select and/or acquire resistance mechanisms. In this context, the clinical application of the MPC is clear: maintaining serum/tissue concentrations in excess of the MPC (within tolerable doses) will require cells to obtain two concurrent resistance mutations for growth and thereby severely restrict the likelihood that resistant mutants will be selected during therapy.

As we learn more about the accumulation of antibiotics in infected tissues, the types of resistance mutants selected by different agents within different drug concentration spectrums, and the concentrations required to inhibit their growth, the practical implications of the MPC will continue to emerge. A recent review of MPC by Blondeau *et al* (32) noted that the development of MPC is a relatively new concept that continues to evolve with every report. Therefore, with such a new concept and relatively limited studies published to date, it may be premature to comment on overstretching the limits of MPC when we do not yet know if the limits have been defined (32). Open debates and discussions will help to develop a greater understanding of fluoroquinolone resistance and the potential impact of the MPC. Based on the development of penicillin-resistant *S. pneumoniae*, clinical validation may come in the form of increased clinical failures and a rapid rise in the rates of resistance. Thus, perhaps the most meaningful clinical question regarding the MPC is whether or not we can afford to take "a wait and see" approach to fluoroquinolone resistance?

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8.0 APPENDIX A

8.1 Solutions and Buffers

10X TE Buffer
Dissolve 12.1 g of Tris Base and 3.72 g of EDTA in 750 ml of distilled water. Bring to pH 8.0 using HCl. Adjust volume to 1 L and autoclave.

10X TBE
Dissolve 90.8 g of Tris Base, 15.4 g of Boric acid and 0.37 g of EDTA in 1000 ml of distilled water and autoclave.

0.5 M EDTA
Add 93.05 g of EDTA to 400 ml of distilled water. Add NaOH pellets one at a time until the EDTA is completely dissolved. Bring the pH to 8.0 by adding 5 M NaOH. Adjust volume to 500 ml and autoclave.

0.1 M Cacodylate Buffer
Combine 16 g of sodium cacodylate (Mol. Wt. 160.0) with 920 ml of distilled water. pH to 7.2 with 0.1 N HCL. Adjust to 1000 ml with distilled water.

10% Sarcosyl
Dissolve 10 g of sodium sarcosinate in 70 ml distilled water. Use of heat will aid in dissolving the sarcosyl. Adjust volume to 100 ml and autoclave.

1% Pulsed-Field Agarose
Add 1.0 g pulsed-field agarose to 100 ml 0.5X TBE. Boil until agarose is dissolved and cool to 50°C before pouring the gel. Allow gel to solidify for a minimum of 30 min before use.

1% Agarose Gel for PCR
Add 0.35 g to 35 ml of TBE Buffer containing ethidium bromide (ETBr). Microwave on high until the agarose is completely dissolved. Pour gel and allow it to solidify for ~30 min.

2% Glutaraldehyde
Add 10 ml of 25% EM grade Glutaraldehyde to 115 ml of 0.1 M cacodylate buffer.

Cell Suspension Buffer
Combine 100 µl of 1 M Tris-HCl (pH 7.2), 200 µl of 1 M NaCl and 1.0 ml of 0.5 M EDTA (pH 8.0). Dilute to 10 ml with sterile distilled water.

ETBr Buffer
Add 50 µl of ETBr at 10 mg/ml to 1 L of TBE Buffer for a final ETBr concentration of 500 µg/ml.

ETBr Solution For Staining a Pulsed Field Gel
Combine 40 µl stock ethidium bromide with 400 ml of distilled of distilled water.

Low Melting Point Agarose
Dissolve 1 g in 100 ml of TE Buffer at pH 8.0. Boil for 1-to-1.5 min and cool to 50-65°C in water bath.

Lysis Buffer
Combine 100 µl of 1 M Tris-HCl (pH 7.2), 500 µl of 1 M NaCl and 1.0 ml of 0.5 M EDTA (pH 8.0). Weigh out 20 mg of deoxycholate and 50 mg of N-Lauroylsarcosine and add to above mixture. Dilute to 10 ml with sterile distilled water.

PFGE Running Buffer
Dilute 300 ml of 10X TBE in 2700 ml of distilled water for a concentration of 1X TBE.

Proteinase K Buffer
Combine 50 ml of 0.5 M EDTA (pH 8.0) and 10 ml of 10% N-Lauroylsarcosine. Adjust pH to 9.0, then dilute to 100 ml with sterile distilled water.

Proteinase K/Proteinase K Buffer
Add 25 µl of 20 mg/ml of Proteinase K to 10 ml of Proteinase K Buffer.

Reynold's Lead Citrate
Mix 1.33 g lead acetate, 1.76 g of sodium citrate and 30 ml of double distilled water that has been boiled to remove CO_2. Shake vigorously for 1 min then place on a magnetic stirrer for 30 min. Add 8 ml of 1 N NaOH. Adjust to 50 ml using boiled deionized water. Mix well.

Skim Milk
Dissolve 200 g of powdered skim milk in 1000 ml of distilled water and autoclave.

TE Buffer
Add 5 ml of 1 M Tris-HCl, pH 8.0, and 8 ml of 0.25 M EDTA, pH 8.0, into 494 ml of distilled water.

Toluidine Blue
Mix 2.5 g of Toluidine Blue, 2.5 g of sodium borate and 250 ml of distilled water for several hr.

Tracking Dye
Add 60 g of sucrose into 100 ml sterile distilled water to make a 60% sucrose mixture. Add 0.25 g of xylene cyanol into 100 ml of sterile distilled water. Add 10 mM Tris at pH 8.0. Combine the 60% sucrose, the 0.25% xylene cyanol.

TSA
Add 40 g to 1 L of distilled water. Autoclave.

Wash Buffer
Combine 10 ml of 1M Tris-HCl (pH 7.6) and 20 ml of 0.5 M EDTA, pH 8.0. Dilute to 100 ml with sterile distilled water.

8.2 APPENDIX B

8.2 Suppliers

8.2.1 Media

Mueller Hinton Broth (MHB)	Becton, Dickinson and Co., Sparks, MD
Todd Hewitt Broth (THB)	Becton, Dickinson and Co., Sparks, MD
Tryptic Soy Agar (TSA) with 5% Sheep Blood	Becton, Dickinson and Co., Sparks, MD

8.2.2 Antimicrobial Agents

Moxifloxacin, ciprofloxacin – Bayer pharmaceutical, West Haven, CT
Levofloxacin – The R. W. Johnson Pharmaceutical Research Institute, Raritan, NJ
Gatifloxacin – Bristol Myers Squibb, Montreal, QC
Grepafloxacin – – Bristol Myers Squibb, Montreal, QC
Trovafloxacin – Pfizer Canada, Kirkland, QC
Garenoxacin - – Bristol Myers Squibb, Montreal, QC
Gemifloxacin – Glaxco Smithkiline pharmaceuticals, Collegeville, PA
Ceftazadime – – Glaxco Smithkiline pharmaceuticals, Collegeville, PA
Gentamiycin – Sigma-Aldrich Co., St. Louis, MO
Tobramycin - Sigma-Aldrich Co., St. Louis, MO

8.2.3 Reagents, Chemicals and Enzymes

25% Glutaraldehyde (EM Grade)	Marivac, St. Laurent, QC
95% Alcohol	Commercial Alcohols Inc., Brampton, ON
Agarose	Invitrogen, Corisbad, CA
Ammonium Hydroxide	BDH Inc., Toronto, ON
Araldite 502	Ted Pella, Inc., Millville, NJ
Boric Acid	BDH Inc., Toronto, ON
Buffer A	New England BioLabs, Mississauga, ON
Buprenorphine	Reckitt and Colman Pharmaceuticals Inc. Richmond,VI
CCCP	Sigma-Aldrich, St. Louis, MO
Deoxycholate Acid	Sigma-Aldrich Co., St. Louis, MO
DMP-30 [Tri(Dimethylaminoethyl Methacrylate)]	Ted Pella, Inc., Millville, NJ
EDTA	Sigma-Aldrich Co., St. Louis, MO
Ethidium Bromide	BioRad Loaboratories, Hercules, CA

Ethanol	Electron Microscopy Sciences, Fort Washington, PA
Eyelube	Sabex, Boucherville, QC
Hydrochloric Acid (HCl)	BDH Inc., Toronto, ON
InstaGene Matrix	BioRad Laboratories, Hercules, CA
Kodak D-19 Developer	Kodak, Saint-Laurent, QC
Kodak Rapid Fixer	Kodak, Saint-Laurent, QC
Ketamine	Warner-Lambert and Co., Bellevelle, ON
Lambda Ladder	New England BioLabs, Mississauga, ON
Lead Acetate	Anachem, Bedfordshire, United Kingdom
Lead Citrate	Ted Pella, Inc., Millville, NJ
Low Melting Point Agarose	BioRad Laboratories, Hercules, CA Sigma Chemical Co., St. Louis, MO
Mass Ladder	Invitrogen, Burlington, ON
N-Laurylsarcosine	Sigma Chemical Co., St. Louis, MO
Oxidase	Fisher scientific, Nepean, ON
Pentobarbital sodium	Bimeda-MTC Animal Health Inc. Cambridge, ON
PCR Ladder	Invitrogen, Carisbad, CA
PCR Primers	Sigma-Genosys, Oakville, ON
PuReTaq Ready-To-Go	Amersham/Pharmacia, Piscataway, NJ
Pfx platinum taq polymerase	Gibco-BRL, Burlington, ON
Proteinase K	Sigma Chemical Co., St. Louis, MO
Pulsed Field Certified Agarose	Sigma Chemical Co., St. Louis, MO
Saline	Baxter, Deerfield, IL
Skim Milk	Becton, Dickinson and Co., Sparks, MD
Slidex Pneumo-Kit	bioMerieux, Marcy-l'Etoile, France
SmaI	New England BioLabs, Mississauga, ON
SpeI	New England BioLabs, Mississauga, ON
Sodium Borate	Fisher Scientific, Nepean, ON
Sodium Cacodylate	Ted Pella, Inc., Millville, NJ
Sodium Chloride (NaCl)	BDH Inc., Toronto, ON
Sodium Citrate	BDH Inc., Toronto, ON
Sodium Hydroxide (NaOH) Pellets	BDH Inc., Toronto, ON
Sucrose	BDH Inc., Toronto, ON
Tris-HCl	Sigma Chemical Co., St. Louis, MO
Uranyl Acetate	Ted Pella, Inc., Millville, NJ
Vetbond tissue adhesive	3M animal care products St. Paul MN
Wizard Kit	Promega, Madison, WI
Xylene Cyanol	BioRad Laboratories, Hercules, CA
Zylazime	Bayer Veterinary Division, Toronto, ON

8.2.4 Disposable Labware

200 µl Pipette Tips	VWR International, Edmonton, AB
Corning Cryovials	Corning Inc., Corning, NY
Cuvettes	Fisher scientific, Nepean, ON
Disposable scalpels	Fisher scientific, Nepean, ON
Falcon multiple well tissue Culture plates	VWR Canlab, Edmonton, AB
Glass Tubes	Fisher scientific, Nepean, ON
Latex Gloves	Fisher scientific, Nepean, ON
McFarland Tubes	Fisher scientific, Nepean, ON
Microcentrifuge Tube	Fisher scientific, Nepean, ON
Microtitre Plates	Sarstedt, Newton, NC
Pasteur Pipettes	Fisher scientific, Nepean, ON
Sterile Plastic Petri Plates	Fisher Fisher scientific, Nepean, ON
Swabs	Fisher scientific, Nepean, ON
Wooden Applicator Sticks	Puritan, Guilford, ME
3cc Monoject Leur Lock Syringe	Sherwood Medical, St. Louis, MO
20 gauge needle	Sherwood Medical, St. Louis, MO

8.2.5 Equipment

20 ul, 200 µl and 1 ml Pipettors	Gilson Company, Inc., Lewis Center, OH
-70°C Freezer	Forma Scientific Inc., Marjetta, OH
Avanti J-E Centrifuge	Beckman Coulter, Palo Alto, CA
CHEF DRIII PFGE System	BioRad Laboratories, Mississauga, ON
Colorimeter	Hach Company, Loveland, CO
DNA theromocycler	MJ Research, Waltham, MA
Forcepts	Fisher scientific, Nepean, ON
Hot Plate/Stirrer-Model 300T	Fisher scientific, Nepean, ON
Homegenizer	Labcor Concord ON
Hibitane soap	Fisher scientific, Nepean, ON
Needle driver	Fisher scientific, Nepean, ON
Pediatric Bactec blood culture bottles	Beckton Dickson, Sparks, MD
PCR Gel Casting Mold	BioRad Laboratories, Mississauga, ON
PFGE Gel Casting Mold	BioRad Laboratories, Mississauga, ON
pH Meter	Corning Inc., Corning, NY
Pulsed Field Gel Casting Apparatus	BioRad Laboratories, Mississauga, ON
Gel Doc 1000 Illuminator	BioRad Laboratories, Mississauga, ON

Microwave Oven	Samsung, Suwon, Korea
pH meter	BioRad Laboratories, Mississauga, ON
Oxygen Incubator	Hotpack Corp., Philadelphia, PA
Rat tail vein catheter	Becton Dickinson Infusion Therapy Sysem Inc., Sandy, UT
Shaking Water Bath	Mandel Scientific Co., Guelph, ON
Spectrophotometer	Pharmacia, Cambridge, United Kingdom
Thermocycler (PCR Express)	Thermo Hybaid, Ashford, Middlesex, United Kingdom
Vortex (Mini-Shaker Model 58)	Fisher scientific, Nepean, ON
Vicryl silk sutures	Ethicon, Sommerville, NJ
Viteck identification/susceptibility Cards	BioMerieux, St. Laurent, QC
Weigh Scale – Mettler PC440	DeltaRange, Zurich, Switzerland

8.2.6 Rats

Male, Sprague Dawley white rats	Charles River, Wilmington, MA