Evaluating Methods Used to Study Low-Level Fluoroquinolone Resistance in *Streptococcus pneumoniae*

By

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A Thesis

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

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Abstract

The purpose of this thesis was to demonstrate that current phenotypic methods fail to identify fluoroquinolone-susceptible and low-level fluoroquinolone-resistant isolates of *Streptococcus pneumoniae* harboring resistance-associated mechanisms, more specifically that current fluoroquinolone susceptible breakpoints for *S. pneumoniae* do not reflect the wild-type population and that the reserpine agar dilution method does not effectively detect fluoroquinolone efflux.

The first objective was to assess the prevalence of *parC* and *gyrA* mutations as well as the presence of an efflux mechanism in fluoroquinolone-susceptible isolates of *S. pneumoniae* with a levofloxacin MIC of 1 μg/mL in order to determine a levofloxacin susceptibility breakpoint that more accurately describes the wild type population than does the current CLSI breakpoint of ≤ 2 μg/mL. This was accomplished by sequencing the QRDRs of *parC* and *gyrA* to identify any mutations and by performing the reserpine agar dilution assay to identify efflux positive isolates.

The second objective was to assess the validity of the reserpine agar dilution assay as an appropriate method of screening for ciprofloxacin efflux in *S. pneumoniae* by comparing the results of the reserpine agar dilution assay to results of ciprofloxacin fluorescence accumulation studies.

The prevalence of *parC* and/or *gyrA* mutations in fluoroquinolone-susceptible isolates was low (0.9% from 1995-1997 and 2.1% in 2003). No fluoroquinolone-susceptible isolates exhibited efflux. These results confirm that a levofloxacin MIC of 1 μg/mL accurately describes the wild-type fully susceptible population.

The reserpine agar dilution assay results were not supported by the fluorescence accumulation studies; thus concluding that the reserpine agar dilution assay is not an appropriate method of screening for efflux in *S. pneumoniae.*

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

ABC	ATP-Binding Cassette
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
bp	Base pair
CCCP	Carbonyl cyanide m-chlorophenylhydrazone
CFU	Colony forming unit
Cipro	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
CROSS	Canadian Respiratory Organism Susceptibility Study
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediamine-tetra-acetic acid
EUCAST	European Committee on Antimicrobial Susceptibility Testing
Kb	Kilobase pairs
MATE	Multidrug and toxic compound extrusion
MDR	Multidrug resistance
MFS	Major facilitator superfamily
MIC	Minimum inhibitory concentration
mL	Millilitre
mM	Millimolar
ng	Nanogram
NWT	Non-wild-type
OD	Optical density
PCR	Polymerase chain reaction
QRDR	Quinolone resistance-determining region
RT-PCR	Reverse transcriptase polymerase chain reaction
SBA	Trypticase soy agar with 5% sheep blood
SMR	Small multidrug resistance
TMS	Transmembrane segment
µg	Microgram(s)
µL	Microlitre(s)
µm	Micrometer(s)
µM	Micromolar
vol/vol	Volume/volume

1. Introduction

1.1. Thesis Scope

As the rates of clinically relevant fluoroquinolone resistance in *Streptococcus pneumoniae* begin to rise, more research has come to focus on the population of isolates exhibiting low-level fluoroquinolone resistance. This thesis highlights the significance of low-level fluoroquinolone resistance in *S. pneumoniae* as a major risk for the emergence of high-level, clinically relevant resistance. In studying the mechanisms that contribute to low-level fluoroquinolone resistance in *S. pneumoniae*, it has become evident that current methods of identifying fluoroquinolone resistance fail to detect low-level resistance.

1.2. *Streptococcus pneumoniae*

1.2.1. Characteristics of the Organism

Since the late 1800's, *S. pneumoniae* has been recognized as a significant human pathogen[1]. *S. pneumoniae* belongs to the mitis species group and is phylogenetically closely related to other members of the viridans group streptococci which are typically considered avirulent normal flora [2]. The mitis group streptococci are Gram-positive, catalase-negative, facultative anaerobes characterized by α-hemolysis when grown on blood agar[2]. In the laboratory *S. pneumoniae* is differentiated from other viridans streptococci by positive bile solubility and optochin sensitivity tests[1, 2]. Under the microscope *S. pneumoniae* typically appears as lancet-shaped diplococci 0.5-1.25 μm in diameter, or when grown in liquid medium as short chains of cocci[1, 3]. It is a nutritionally fastidious organism and displays optimal growth when cultured on media containing 5% sheep blood and incubated at 35°-37°C in 3%-5% CO_2[3].

The vast majority of clinical isolates of *S. pneumoniae* possess a capsule. To date 90 serotypes have been identified based on capsular polysaccharides[1]. The capsule is a major virulence factor and plays an important role in evading phagocytosis. Other major virulence factors include pneumolysin, autolysin, pneumococcal surface protein A, pneumococcal surface adhesin A and neuraminidase which are all universally expressed in pneumococci[1, 3, 4].

1.2.2. Epidemiology

The pneumococcus is a uniquely human pathogen that finds its niche colonizing the upper respiratory tract of 5-50% of the healthy population[5]. Colonization rates tend to vary with age. In a single attempt at culturing, 5-10% of healthy adults and 20-40% of healthy children yield pneumococci and these percentages increase with repeated culture attempts[1]. There also appears to be a seasonal effect on colonization rates that corresponds to the seasonal occurrence of viral respiratory infections which peak in mid-winter[3].

Transmission of *S. pneumoniae* occurs from person to person as a result of extensive close contact; infection is nonetheless not considered contagious as transmission may result solely in colonization without progression to disease[1]. Attendance of day care centres presents as a risk factor for transmission and infection amongst toddlers. Similarly, epidemics among adults are typically a result of crowded living conditions such as those found in prisons, military camps and nursing homes[1].

S. pneumoniae is the leading cause of community acquired pneumonia and a common cause of other respiratory tract infections such as acute exacerbations of chronic

bronchitis, otitis media and sinusitis[5]. It is also a major cause of invasive disease including meningitis and bacteremia and causes significant morbidity and mortality worldwide[1]. Bacteremia in adults typically results as a progression of pneumonia. It is estimated that one case of bacteremic pneumonia occurs for every 3-4 cases of non-bacteremic pneumonia[1]. Incidence of pneumococcal pneumonia in the United States is estimated at 25 and 280 cases per 100,000 young adults and elderly persons, respectively. Population based studies show highest incidence of invasive pneumococcal disease in infants under 2 years of age and in adults over 65 years old. Representative data of pneumococcal bacteremia cite an incidence in the United States of 160 cases per 100,000 infants, 5 cases per 100,000 young adults, and 70 cases per 100,000 elderly persons over 70 years old[1]. Some reports suggest a decrease in incidence of pneumococcal disease which could be a result of the implementation of vaccine programs. In North America two vaccines are available covering serotypes that account for the majority (85-90%) of invasive disease[5]. The 23-valent polysaccharide vaccine is recommended for the elderly (over 65 years of age) and immunocompromised persons[5]. The second pneumococcal vaccine is a 7-valent conjugate vaccine and is recommended for young children[5].

Treatment of respiratory tract infections is most often done empirically and thus requires the use of antibiotics that give adequate coverage of the major causative agents[6]. Historically, first line treatment of such infections has been limited to penicillin and other β-lactams due to nearly universal penicillin susceptibility of *S. pneumoniae*[7]. In North America prior to 1989, prevalence of penicillin resistance in *S. pneumoniae* was less than 5%[8]. In 2003, the overall rate of penicillin non-susceptibility in the United States was found to be 34.2%[9]. Varying by location, reports as high as 50% penicillin non-

susceptible have been reported in recent years. With the increased resistance to β-lactams, a dramatic increase has also been noted in many non-β-lactam antibiotics. Resistance rates for macrolides, trimethoprim-sulfamethoxazole, tetracyclines and chloramphenicol have been reported as high as 30%, 40%, 17% and 12% respectively[9]. Canada has followed a similar trend in increasing resistance to both β-lactams and non-β-lactam antibiotics. In 2004 penicillin non-susceptibility was 25.3% in Canada and macrolide resistance had reached 13.4%.

1.3. Fluoroquinolones

1.3.1. Discovery and Development

In 1962 during the production of chloroquine, the formation of the accidental by-product nalidixic acid brought forth the development of a new class of antimicrobial agents: the quinolones [10, 11]. The nalidixic acid backbone has served as a template for the development of many 4-quinolone molecules that display excellent antimicrobial activity against a wide range of both Gram-negative and Gram-positive organisms [10, 11]. In the last two decades over 10,000 compounds derived from the pharmacophore shown in figure 1.1 have been developed and have undergone initial screening[12]. Several classification schemes exist grouping quinolones by chemical structure, structure-activity relationships, *in vitro* spectra of activity and clinical efficacy[13]. The classification scheme seen in figure 1.2 groups quinolones into 4 generations based on their respective microbiologic spectra of activity. Though useful in the treatment of urinary tract infections, early quinolones, showed little potential as a therapy for systemic infections due to their limited spectrum of activity and poor tissue penetration [10, 11]. These first generation quinolones including

Figure 1.1 **Quinolone pharmacophore and structure activity relationships of the various side chains.**

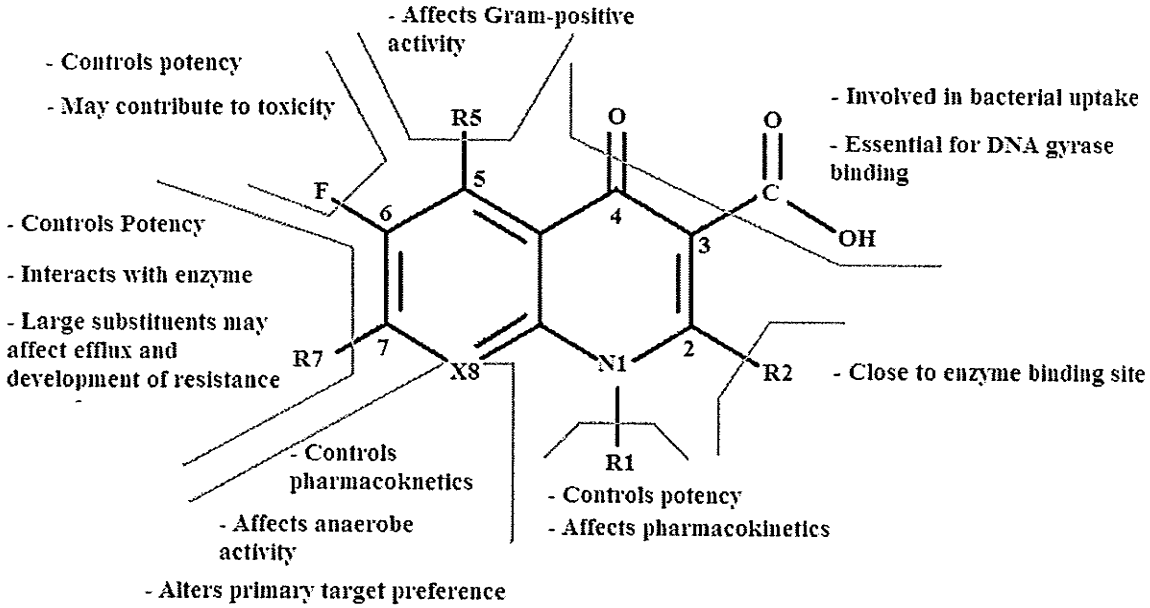
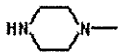
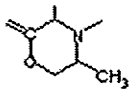
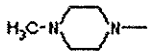
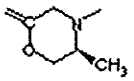
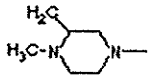
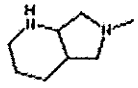
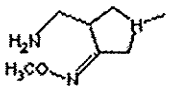
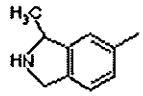

- Affects Gram-positive activity
- Controls potency
- May contribute to toxicity
- Involved in bacterial uptake
- Essential for DNA gyrase binding
- Controls Potency
- Interacts with enzyme
- Large substituents may affect efflux and development of resistance
- Close to enzyme binding site
- Controls pharmacoknetics
- Affects anaerobe activity
- Alters primary target preference
- Controls potency
- Affects pharmacokinetics
R5
O
O
F
5
6
4
C
OH
3
R7
7
X8
N1
2
R2
R1

Figure 1.2 **Classification and side chain structures of various quinolones. Adapted from Van Bambeke *et al* (2005)[13].**

Generation	Drug Name	X	R8	R1	R5	R6	R7
1	Nalidixic acid	N		$-CH_2-CH_3$	H	H	$-CH_3$
2	Norfloxacin	C	H	$-CH_2-CH_3$	H	F	
	Ciprofloxacin	C	H		H	F	
	Ofloxacin				H	F	
3	Levofloxacin				H	F	
	Gatifloxacin	C	$-O-CH_3$		H	F	
	Moxifloxacin	C	$-O-CH_3$		H	F	
	Gemifloxacin	N			H	F	
4	Garenoxacin	C	$-O-CHF_2$		H	H	

nalidixic acid, cinoxacin and pipemidic acid had good activity against Gram-negative aerobes but were ineffective against Gram-positive organisms[12-14].

In the early 1980's the second generation quinolones ciprofloxacin and norfloxacin were developed[13]. These compounds were characterized by the addition of a fluorine at position C-6 and a piperazine at C-7 and became known as the fluoroquinolones[10, 11]. Ciprofloxacin and norfloxacin displayed good penetration of the pulmonary tissues and fluids, have a broad spectrum of activity, and thus continue to serve as an effective treatment for respiratory infections due to organisms such as *Haemophilus influenzae, Moraxella catarrhalis* and *Chlamydia pneumoniae*[15,16]. The second generation quinolones however remain inappropriate treatments for infections caused by *S. pneumoniae* due to the high probability of selection for resistance resulting in treatment failure[13, 15].

The third generation fluoroquinolones marked a major advancement in theraputics of community-acquired respiratory tract infections, as these fluoroquinolones are characterized by an increased potency against *S. pneumoniae*. Levofloxacin, moxifloxacin, gatifloxacin and gemifloxacin are considered the respiratory fluoroquinolones[10, 11, 13].

As a growing understanding of the structure-activity relationships has developed, compounds have been optimized to maintain the clinical efficacy and reduced selection of resistance of third generation compounds but have improved safety due to the removal of the fluorine atom at position 6 which is believed to be involved in genotoxicity[13]. The positions optimized include a cyclopropyl at position 1, a methoxy at position 8 and a pyrrolidine, substituted pyrrlidine or substituted piperazine at position 7. These fourth

generation quinolones are referred to as the non-fluorinated quinolones or the des-fluoroquinolones. Garenoxacin was the first of the fourth generation quinolones developed[13].

1.3.2. Mechanism of Action

1.3.2.1. Drug Uptake

Fluoroquinolone action requires a sufficient amount of accumulation within the cell in order to act on the molecular target. To enter the bacterial cell, fluoroquinolones must cross the lipid bilayer that forms the cytoplasmic membrane [17, 18]. Fluoroquinolones are capable of simply diffusing across the lipid bilayer of biological membranes and achieve an intracellular distribution[17]. In Gram-negative organisms, the outer membrane presents an additional barrier for fluoroquinolone entry into the cell; however, fluoroquinolones are sufficiently small to pass through porins in the outer membrane [17, 18].

In Gram-positive organisms such as *S. pneumoniae,* the amount of drug which can accumulate inside the cell is dependent upon several factors including the rate of diffusion across the membrane, the final distribution at equilibrium, and the organism's ability to pump the drug out of the cell, termed fluoroquinolone efflux [17]. All three factors are determined by the various functional groups attached to the basic fluoroquinolone molecule, though fluoroquinolone efflux is often strain dependent [10]. The rate of diffusion across the membrane and the final equilibrium in the absence of efflux are functions of the protonation levels at physiological pH [17, 18]. Since biological membranes are essentially impermeable to charged ions, it is only the uncharged form of the

fluoroquinolone molecule that is capable of entering the cell [17, 18]. Although amphoteric fluoroquinolones tend to have a greater proportion of the uncharged form at pH 7 and would be predicted to enter cells more rapidly, this does not have an impact on comparative drug efficacy, as influx of all fluoroquinolones is very rapid and equilibrium is normally attained in a matter of seconds [17]. Fluoroquinolone activity is known to be mainly a function of its affinity for the molecular targets.

1.3.2.2. Molecular Targets: DNA Gyrase and Topoisomerase IV

Fluoroquinolones act by inhibiting DNA synthesis, targeting the bacterial type II topoisomerases, DNA gyrase and topoisomerase IV[19, 20]. DNA topoisomerases are enzymes that alter the topological state of DNA by generating transient breaks along the DNA backbone and passing one section of double-stranded DNA through another [21, 22]. These enzymes are essential for several cellular processes including DNA replication and transcription. Type II topoisomerases are characterized by their ability to form double-stranded breaks in DNA in a reaction coupled to ATP binding and hydrolysis[21, 22]. Though DNA gyrase and topoisomerase IV perform similar functions, several key differences exist. DNA gyrase acts ahead of the replication fork during replication and transcription facilitating movement by adding negative supercoils[19, 21]. Positively supercoiled DNA is wrapped around the enzyme generating the negative supercoils. In contrast, topoisomerase IV acts behind the replication fork where it binds to crosslinks in the DNA[19, 21]. DNA is not wrapped around the enzyme. The major role of topoisomerase IV is the decatenation of newly synthesized DNA strands, though it also functions in creating positive supercoils[19, 21, 22].

DNA gyrase and topoisomerase IV are heterotetrameric enzymes of the form $GyrA_2GyrB_2$ and $ParC_2ParE_2$ respectively. The GyrA and ParC subunits are present as dimers that bind DNA and carry out cleavage and religation reactions[19, 21, 22]. During replication an active site tyrosine on either enzyme (Tyr122 on GyrA and Tyr120 on ParC) covalently binds to the DNA phosphate backbone. This induces a site-specific cleavage creating a staggered cut with 3' hydroxyl termini and 4 bp 5' overhangs that are bound to the catalytic enzyme[21, 22].

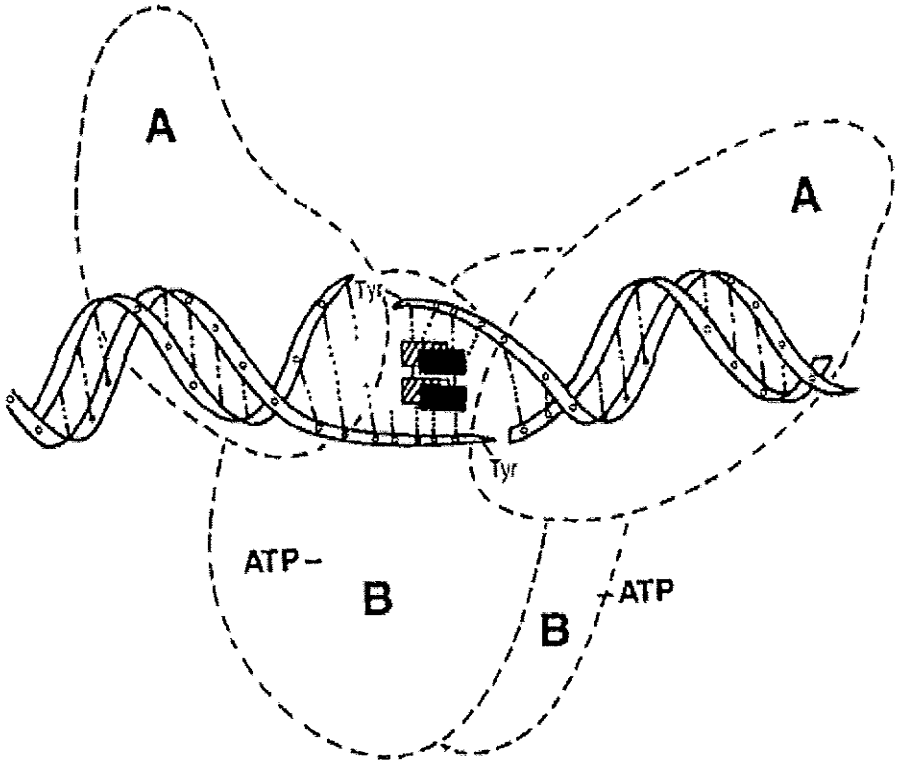
The GyrB and ParE subunits are involved in ATP binding and hydrolysis. Present as monomers, these subunits dimerize upon binding of ATP. Conformational changes then catalyze the enzymatic reaction. ATP hydrolysis allows the GyrB and ParE subunits to return to their monomeric forms preparing the enzyme for a second round of action[21, 22].

1.3.2.3. Bacteriostatic and Bactericidal Activities

Fluoroquinolones bind to complexes of DNA and topoisomerase IV or DNA and DNA gyrase[13, 19, 20, 23-26]. The proposed model for binding as described by Shen *et al* is outlined in figure 1.3[27]. As a general rule, the fluoroquinolone's primary target in Gram-negative organisms is DNA gyrase and in Gram-positive organisms is topoisomerase IV[20, 23]; although this does not hold true for the respiratory fluoroquinolones and *S. pneumoniae* as the primary target varies by agent[28, 29].

Irrespective of which enzyme the quinolone targets, the quinolone-DNA-enzyme complex created is called the cleavable complex[23, 24, 26]. Upon formation of the cleavable complex, a conformational change occurs, the enzyme breaks the DNA and the quinolone

Figure 1.3 **Proposed model for fluoroquinolone binding as a complex with DNA and DNA gyrase. Four quinolone molecules are represented by the four rectangles bound to the DNA. Adapted from Shen *et al* (1996)[27].**


A
A
Tyr
Tyr
ATP –
B
B
+ ATP

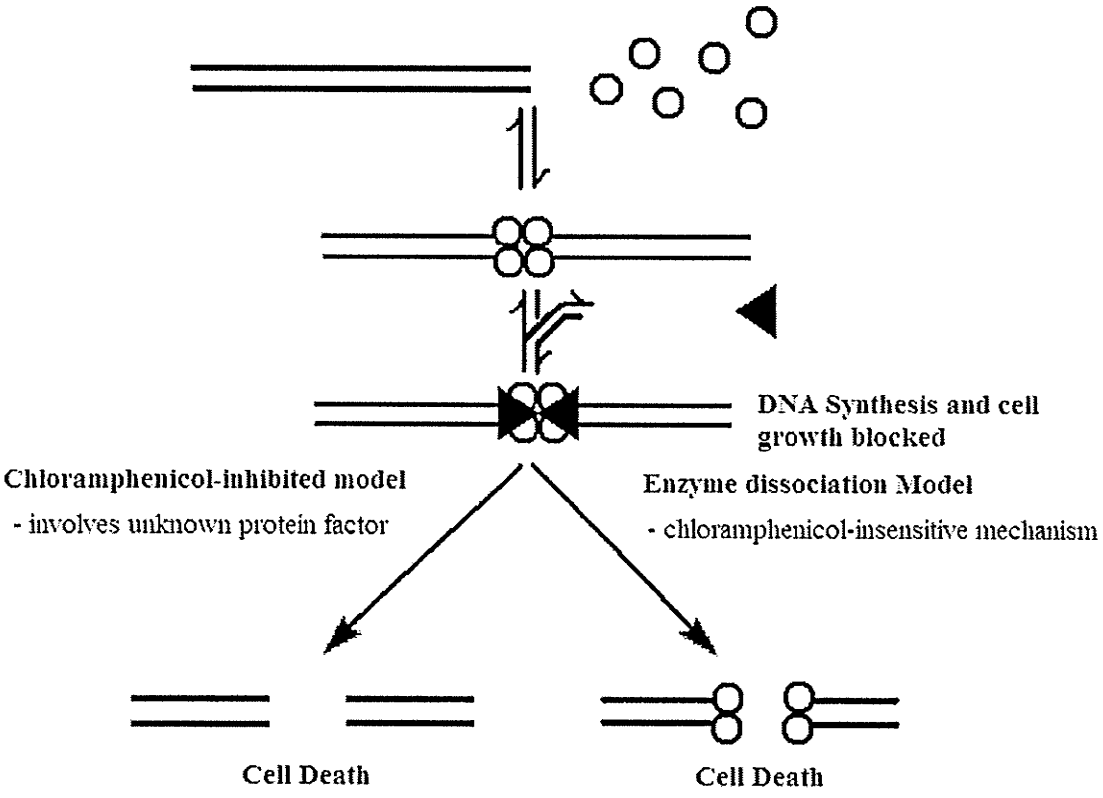
prevents re-ligation. The quinolone stabilizes the enzyme on the DNA and halts replication[20, 23, 25]. At low fluoroquinolone concentrations, cleavable complex formation is reversible and is responsible for the bacteriostatic action of fluoroquinolones. Higher concentrations of fluoroquinolone are required to induce lethal effects[20, 23, 30].

The specific events occurring during fluoroquinolone-mediated cell death remain unknown. The accepted theory is that cell death occurs when the ends of the broken DNA are released and the SOS repair system is induced[19, 23]. Two main hypotheses have been proposed to explain the mechanisms by which cell death occurs. These hypotheses are outlined in figure 1.4. At high quinolone concentrations the cleavable complex is formed and the DNA is broken. At this point, either free DNA ends are released or the enzyme subunits dissociate and remain bound to the ends of the broken DNA[23].

The first theory proposes that lethal activity resulting in the release of free DNA ends can be inhibited by the protein synthesis inhibitor chloramphenicol. In this reaction, a protein factor is required to dissemble the cleavable complex. In the absence of the protein factor (i.e. in the presence of chloramphenicol), the broken DNA is not released and cell death does not occur[23]. This model dictates that all cleavable complexes present on the chromosome are capable of releasing broken DNA. The concentration dependence of fluoroquinolone lethal action is consistent with this model. Although the lethal activity of nalidixic acid can be completely inhibited by the addition of chloramphenicol, this is not the case for many fluoroquinolones. The enzyme dissociation model is proposed as an alternative to the chloramphenicol-sensitive mechanism.

The enzyme dissociation model suggests that only those complexes associated with replication forks are affected. As replication proceeds, the replication fork advances

Figure 1.4 **Schematic representation of proposed bactericidal mechanisms of fluoroquinolone induced cell death. Bacterial dsDNA is represented by double lines. Circles represent DNA gyrase or topoisomerase IV subunits. Black triangles represent quinolone molecules. Adapted from Chen *et al* (1996)[23].**


DNA Synthesis and cell growth blocked
Chloramphenicol-inhibited model
- involves unknown protein factor
Enzyme dissociation Model
- chloramphenicol-insensitive mechanism
Cell Death
Cell Death

along the chromosome and collides with the stabilized cleavable complex. This results in the release of broken DNA still bound to the enzyme[19, 23, 30]. Analysis of the time dependency of fluoroquinolone action supports this model. Studies show that the inhibition of DNA gyrase activity by fluoroquinolones occurs more rapidly than inhibition of topoisomerase IV activity. This finding is consistent with the location of the enzymes with respect to the replication fork; however, there is no explanation for the concentration dependent activity of fluoroquinolones[23].

1.4. Mechanisms of Resistance

In general, there are three strategies bacteria employ to achieve antimicrobial resistance: drug inactivation/modification, lowered intracellular drug concentrations, and target site alterations[31]. Drug inactivation is typically achieved by drug-modifying enzymes produced by the organism in response to natural antimicrobial compounds found in the environment. Fluoroquinolones are a class of synthetic antimicrobials and it is therefore unlikely that organisms would possess genes encoding enzymes that would specifically target and modify these compounds. Fluoroquinolone resistance can however be achieved by either of the two latter mechanisms [13, 20, 31-34]

Many organisms are capable of decreasing their intracellular fluoroquinolone levels by actively pumping the compounds out of the cell[35]. This strategy, termed efflux, lowers the ability of the fluoroquinolone to kill the organism because fluoroquinolones act in a concentration dependent manner[35].

Alternatively, organisms may develop resistance as a result of a mutation in the fluoroquinolone's molecular target[20]. Mutations in the molecular targets which decrease

the binding affinity of the drug, without compensating the viability of the organism, can effectively lead to clinically relevant levels of resistance[36].

1.4.1. Target Site Mutations

The most common mutations in *parC* and *gyrA* that result in amino acid substitutions known to be associated with fluoroquinolone resistance in *S. pneumoniae* occur in the quinolone-resistance determining regions (QRDRs) of the respective enzyme subunits[30, 33, 34]. The QRDRs of the ParC and GyrA subunits are localized at the amino terminus of each respective protein; this is the catalytic domain containing the active site tyrosine. Amino acid substitutions in the QRDRs of ParC and GyrA are capable of reducing the binding affinity of the fluoroquinolone for the enzyme[30].

Although less frequent than *parC* and *gyrA* mutations, resistance associated mutations may also occur in *parE* and *gyrB*[30]. Amino acid substitutions in these subunits tend to occur in the mid region of the proteins, within the domain involved in subunit interaction[30]. The most common amino acid substitutions associated with resistance are listed in Table 1.1.

QRDR mutations are acquired in a stepwise fashion. Typically, a primary mutation occurs in *parC* at a rate of 1 in 10^7 cells and usually leads to a two-fold increase in ciprofloxacin MIC [37]. The second-step mutation in *gyrA* occurs at a rate of 1 in 10^5 cells and results in a higher level of fluoroquinolone resistance (usually 8-32 fold increase in MIC) [37]. Although resistance to ciprofloxacin in *S. pneumoniae* can be achieved by the acquisition of a single-step *parC* mutation, clinically relevant levels of resistance to the respiratory fluoroquinolones typically requires mutations in both *parC*

and *gyrA*[12]. Single-step mutations may also occur in *gyrA,* the mutations acquired depend largely on the primary target of the agent used for selection. Clinical isolates with single *gyrA* mutations are encountered much less frequently than isolates with single *parC* mutations.

Table 1.1 Amino acid substitutions known to be associated with fluoroquinolone resistance in *S. pneumoniae.*

GyrA	GyrB	ParC	ParE
Ser81Phe	Glu474Lys	Asp78Ala	Asp435Asn
Ser81Tyr		Asp78Asn	Pro454Ser
Glu85Gly		Ser79Ala	
Glu85Lys		Ser79Arg	
		Ser79Leu	
		Ser79Phe	
		Ser79Tyr	
		Ser80Phe	
		Asp83Ala	
		Asp83Asn	
		Asp83Gly	
		Asp83His	
		Asp83Tyr	
		Asp83Val	
		Gln90His	

1.4.2. Efflux

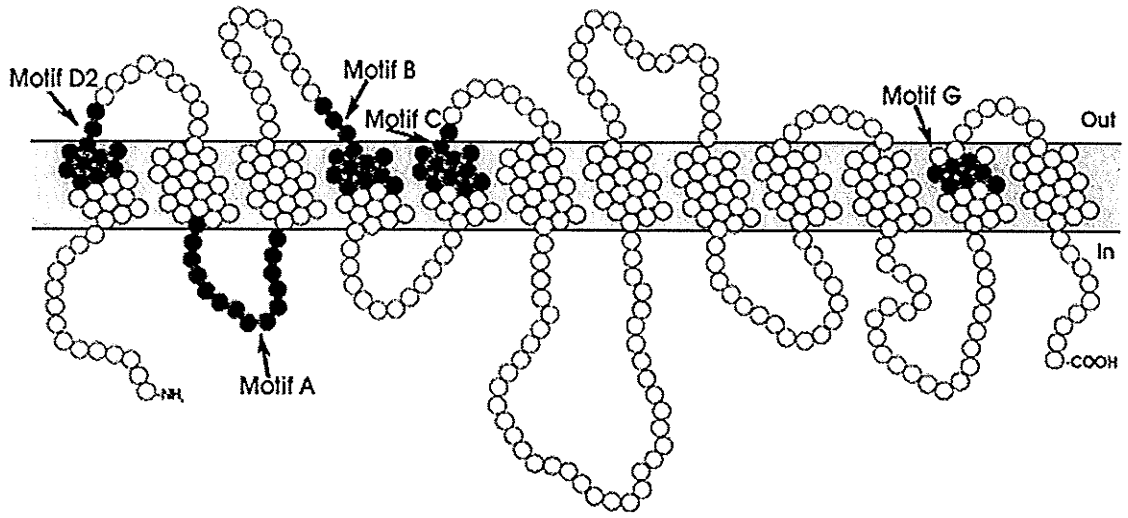
Fluoroquinolone efflux pumps are endogenous pumps present in the organism for reasons other than drug efflux[35]. These pumps serve to protect the cell from a wide variety of toxins and are capable of transporting many toxic compounds such as ethidium bromide, chloramphenicol and rhodamine 6G[35, 38]. Transporters responsible for drug efflux are currently divided into five major classes based on structural information and bioenergetic properties. These include the ATP-binding cassette (ABC) superfamily, major facilitator superfamily (MFS), multidrug and toxic compound extrusion (MATE) family, small multidrug resistance (SMR) family and resistance-nodulation-cell division (RND) superfamily transporters[35, 38-40]. Each class includes transporters responsible for the uptake of ions and essential nutrients as well as the excretion of toxic compounds and metabolic end products[35]. While multi-component efflux pumps, such as those belonging to the RND superfamily, are common in Gram-negative organisms[38], the predominant pumps in Gram-positive organisms are single-component MFS transporters[40]. MFS pumps are characterized by their 12 or 14 transmembrane segments (TMS) and can function as symporters or antiporters that are driven by an electrochemical gradient, usually the proton motive force[35, 39, 40]. There are two mechanisms by which MFS efflux pumps may contribute to resistance: inducible overexpression and constitutive overexpression. Inducible overexpression most likely involves a regulator protein, while constitutive overexpression is thought to be due to mutations upstream of the start codon[31].

1.4.2.1. Efflux in Gram-Positives: NorA and Bmr

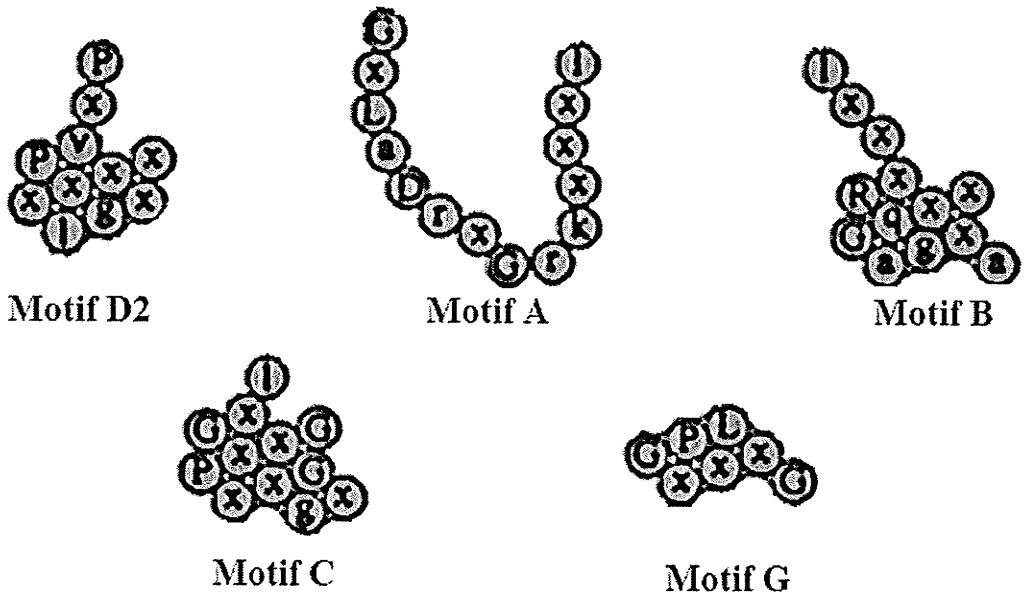
Efflux of fluoroquinolones is well documented in several Gram-positive organisms such as *Staphylococcus aureus* and *Bacillus subtilis,* where NorA and Bmr are the predominant fluoroquinolone efflux pumps respectively[40-42]. These pumps belong to the MFS of transporters which are characterized by 12 membrane spanning domains[40, 41]. The 12-TMS MFS efflux pumps are believed to have evolved from a gene duplication event of a gene encoding a protein with six membrane-spanning regions[43]. The amino terminal half of MFS pumps appears to be more highly conserved among various pumps than does the carboxy terminal half leading to the belief that the amino terminus is most likely involved in proton transport, while the carboxy terminus determines substrate specificity[43]. There are also several highly conserved motifs (figure 1.5) among all MFS pumps, which are related to function. Motif A encompasses a β-turn that is responsible for the reversible conformation change that opens and closes the channel of the pump. Motif B has a highly conserved arginine that is believed to play a major role in proton transport. Motif C most likely plays a role in determining transport direction, as this motif is highly conserved among MFS antiporters, but is absent among symporters[43]. These efflux pumps are driven by the proton motive force. Compounds capable of disrupting the proton motive force, such as carbonyl cyanide m-chlorophenylhydrazone (CCCP), can act as efflux inhibitors[44]. CCCP is only capable of inhibiting pumps driven by the proton motive force and therefore does not inhibit ABC transports. Reserpine, a plant alkaloid, has been shown to inhibit both MFS and ABC pumps. In *B. subtilis* reserpine interacts directly with the Bmr protein. A three amino acid binding pocket is formed of phenylalanine 143, valine 286 and phenylalanine 306[44]. Mutations in position 286 are

Figure 1.5 **Schematic representation of a 12-TMS transporter of the major facilitator superfamily.**
A) Entire transporter. B) Enlarged view of conserved motifs. The consensus sequences of the motifs were determined by alignment of amino acid sequences of transporters found in *B. subtilis*, *Escherichia coli*, *S. aureus* and *Mycobacterium tuberculosis*. X represents any amino acid. Capital letters represent amino acids that occurred in 70% of the sequences examined. Lowercase letters represent amino acids that occurred in 40% of sequences.

A.


Motif D2
Motif B
Motif C
Motif G
Out
In
Motif A
NH2
COOH

B.


Motif D2
Motif A
Motif B
Motif C
Motif G

associated with lower binding affinity of reserpine. NorA has a leucine in position 286 however the other two of the three amino acids present in the binding pocket are conserved[44].

Unlike the MDR efflux pumps of Gram-negative organisms, NorA and Bmr only function in the transport of a single clinically relevant drug class, the fluoroquinolones[40]. They are nonetheless considered MDR pumps due to their ability to transport a wide range of structurally unrelated substrates including many antiseptics, biocides, disinfectants and dyes[35, 40, 43]. In the laboratory, investigators take advantage of the pump's wide range of substrates. Quantitation of accumulation to study efflux is facilitated using visible dyes in place of radiolabelled compounds, as well, induction of efflux can be tested against by a wide variety of substrates[45, 46]. Induction is also an important property to consider in the clinical setting, as continual use of antiseptics and disinfectants may induce overexpression of efflux pumps. Alternatively, excessive use of biocides and disinfectants may aid in the selection of resistant strains[47].

1.4.2.2. Current Methods of Studying Efflux

An organism may possess many efflux pumps that in turn may have broad and overlapping substrate spectrums[35, 40, 48]; each pump may be differentially regulated by local regulators, global regulators and/or two-component systems[39, 45]. Thus, studying accumulation and efflux requires the use of a range of techniques that are each useful for investigating a specific aspect of accumulation.

There are two commonly used methods of screening organisms for fluoroquinolone efflux: the reserpine susceptibility assay and the ethidium bromide

assay[49, 50]. In the former method, fluoroquinolone MICs are measured in the presence and absence of reserpine. A decrease in the ciprofloxacin MIC in the presence of reserpine indicates active ciprofloxacin efflux[51]. This is a relatively quick method for screening for the presence of a reserpine-inhibited fluoroquinolone efflux mechanism and can be performed on many isolates simultaneously. The method has the potential to measure the contribution of efflux to the organism's level of resistance; however, this method is not well standardized and makes a major assumption that the effect reserpine has on susceptibility is due to efflux pump inhibition. In using this assay for *S. pneumoniae* we must assume that reserpine is acting as an inhibitor of fluoroquinolone efflux and this has not yet been demonstrated.

The latter screening method is the ethidium bromide assay. Ethidium bromide is a mutagen commonly used for visualizing nucleic acids because its fluorescence is greatly increased when bound to double stranded DNA[52]. Several of the well characterized fluoroquinolone efflux pumps, including NorA and Bmr of *S. aureus* and *B. subtilis* respectively, also have ethidium bromide as a substrate [51]. Ethidium bromide accumulation is directly proportional to the level of fluorescence. Efflux of ethidium bromide can be assessed based on the change in accumulation rate before and after the addition of CCCP[50]. In an isolate effluxing ethidium bromide via a proton gradient driven efflux pump, the accumulation rate prior to the addition of CCCP should be less than accumulation rate after the addition of CCCP[52]. Alternatively ethidium bromide efflux can be measured directly as a decrease in percentage of fluorescence compared to initial fluorescence over time. The ethidium bromide accumulation and efflux assays are relatively simple and quick compared to measuring accumulation of fluoroquinolones;

however, they rely on the assumption that the efflux pump being assessed has both fluoroquinolones and ethidium bromide as substrates. It may be a poor indicator of fluoroquinolone efflux if a pump has ethidium bromide as a substrate but does not efflux any of the fluoroquinolones.

Drug accumulation studies have typically been performed using radioactively labeled compounds[46]. Radioactivity is proportional to drug accumulation and is measured by a scintillation counter[46]. Since fluoroquinolones are naturally fluorescing compounds the use of radioactivity is not necessary. A fluorescence accumulation assay has been developed to measure accumulation as a function of fluorescence[46]. The assay is significantly more labor intensive than the ethidium bromide accumulation assay due to the comparative fluorescent properties of fluoroquinolones and ethidium bromide.

Efflux-mediated fluoroquinolone resistance is typically due to increased expression of an endogenous pump[40, 53]. Efflux pump gene expression levels can be assessed in wild type and suspected efflux positive isolates by methods such northern blot analysis, quantitative competitive or semi-quantitative reverse transcriptase PCR (RT-PCR)[42, 47, 53, 54]. Both of these methods are capable of quantitating cellular RNA. The expression data is expected to correlate to the accumulation data if the pump being studied is in fact contributing to fluoroquinolone efflux.

Mutant strains are often used to assess the contribution of a particular pump to fluoroquinolone efflux[53, 55]. An efflux pump knockout mutant from of an isolate exhibiting efflux-mediated resistance would have increased fluoroquinolone susceptibility or accumulation would be restored to wild type levels; however, many organisms possess a variety of potential efflux pumps and knocking out a single pump

may not have a significant impact on accumulation. Alternatively, cloning an efflux pump gene with a constitutive promoter into a wild-type strain to overexpress the gene should decrease susceptibility or increase accumulation [47]. Mutating regulators of gene expression may also assist in determining which regulators are involved and to what extent they may contribute to overexpression and efflux-mediated resistance [45].

None of the techniques listed used alone offers a comprehensive assessment of the mechanism of fluoroquinolone efflux in an organism. It is important to use a combination of the methods to confirm or assess the validity of results obtained by one method alone.

1.4.2.3. Evidence of Fluoroquinolone Efflux in *S. pneumoniae*

It is well documented that susceptibility to fluoroquinolones and ethidium bromide in certain strains of *S. pneumoniae* is increased in the presence of reserpine. In 1997 Baranova and Neyfakh generated a laboratory mutant that exhibited increased MICs to ethidium bromide, norfloxacin and ciprofloxacin compared to its parent strain[56]. In the presence of reserpine, MICs of all three compounds were restored to wild type values. Ethidium bromide accumulation and efflux were assessed. The rate of accumulation was shown to be significantly increased in the presence of reserpine. Similarly, efflux was completely inhibited in the presence of reserpine and was found to be decreased in the presence of CCCP[56]. These results support the idea that a reserpine-sensitive energy-dependent multi-drug efflux mechanism capable of pumping out ethidium bromide, norfloxacin and ciprofloxacin was present in *S. pneumoniae*.

In 1999 Gill *et al* reported a putative MFS efflux pump in *S. pneumoniae*, PmrA (pneumococcal multidrug resistance protein), which displayed 24% overall sequence

homology with both NorA and Bmr[57]. PmrA was cloned into and overexpressed in the laboratory strain R6 to generate an efflux mutant R6N. Ethidium bromide accumulation was decreased and accumulation was increased in R6N compared to R6. MICs to norfloxacin, ciprofloxacin, ethidium bromide and acriflavine were also elevated in the mutant strain compared to the parent strain. Moxifloxacin and sparfloxacin MICs were not affected. When PmrA was insertionally inactivated in R6N, accumulation and susceptibility were restored[57]. Subsequently, Piddock *et al* found that clinical isolates exhibiting decreased accumulation and a similar reserpine-sensitive phenotype to R6N did not necessarily express *pmr*A to a greater degree than wild type isolates[53]. Genome sequencing of *S. pneumoniae* has identified numerous putative MFS and ABC transporter genes. There are over 70 putative ABC transporter genes that have no assigned function[52].

Despite the lack of understanding of the specific details involved in fluoroquinolone efflux in *S. pneumoniae*, it is generally accepted that efflux plays a significant role in contributing to low-level fluoroquinolone resistance.

1.5. Defining Resistance

Generally, an organism is considered clinically resistant if an infection caused by this organism is likely to result in bacteriological and clinical failure using standard antimicrobial dosing[58]. Various organizations have devised systems for defining antimicrobial resistance. The Clinical and Laboratory Standards Institute (CLSI) has designed a set of fluoroquinolone breakpoints for *S. pneumoniae* based on a variety of factors including frequency distributions, clinical data and pharmacokinetic-

pharmacodynamic properties. These breakpoints, which designate populations as susceptible (S), intermediate (I) and resistant (R), are designed to assist in predicting bacteriological eradication and clinical success.

The European Committee on Antimicrobial Susceptibility Testing (EUCAST) has addressed the issue of developing resistance by defining microbiological resistance and establishing epidemiological cut-off values in addition to clinical breakpoints[59]. Microbiological resistance distinguishes organisms as wild-type or non-wild type (NWT). An organism is considered NWT for a species by the presence of an acquired or mutational resistance mechanism for a specified drug. The EUCAST epidemiological cut-off values were devised with the goal of monitoring the emergence of resistance [59]. These cut-off values are more sensitive than the clinical breakpoint values in identifying decreased susceptibility.

The work presented in this thesis follows from a larger body of work characterizing fluoroquinolone resistant isolates of *S. pneumoniae*. Although breakpoints are not officially designated for ciprofloxacin and *S. pneumoniae,* for surveillance purposes ciprofloxacin is used as a marker for monitoring fluoroquinolone resistance. Isolates with MICs for ciprofloxacin $\geq$ 4 μg/mL are considered resistant. Isolates with MICs of 4 and 8 μg/mL are deemed as exhibiting low-level resistance and isolates with MICs $\geq$ 16 μg/mL as exhibiting high-level resistance. This work focuses on two groups of isolates, those deemed susceptible according to CLSI levofloxacin breakpoints, and those with low-level ciprofloxacin resistance (MICs of 4 and 8 μg/mL).

1.6. Rational and Objectives

In order to fully understand the development of fluoroquinolone resistance in a population it is crucial to have an understanding of how selection for resistance occurs within the organism. It is well documented that *in vitro* second step *gyrA* mutations are acquired more rapidly than the primary *parC* mutation[37]. The *in vivo* consequences of this fact may be fatal when an infection caused by an isolate with a first-step *parC* mutation is treated with a fluoroquinolone. In recent years, despite the continued low rate of fluoroquinolone resistance in *S. pneumoniae* in Canada, (levofloxacin resistance ~1.9% in 2004 [60]), reports of fluoroquinolone treatment failure with *S. pneumoniae* have been increasing [61, 62]. Although recent reviews have examined the patient factors involved in predicting fluoroquinolone treatment failure and implicate residency of long-term care facilities, hospitalization, and history of fluoroquinolone use as primary risk factors, the underlying cause of many treatment failures is the presence of NWT isolates of *S. pneumoniae* possessing first-step *parC* mutations which rapidly develop a *gyrA* mutation upon treatment with a fluoroquinolone [62]. Several studies have investigated the prevalence of first-step *parC* mutations in levofloxacin-susceptible isolates of *S. pneumoniae* in North America and reports range from a 59% to a 71% prevalence of isolates with a levofloxacin MIC of 2 μg/mL possessing *parC* mutations [4, 63]. Such a high prevalence of NWT isolates in the supposedly susceptible population is alarming when considering the potential risk of selecting for high-level resistance.

Of major concern is the application of inappropriately high breakpoints with fluoroquinolones and *S. pneumoniae* selecting for an increasing number of isolates possessing first-step mutations. These NWT isolates pose an increased risk of treatment

failure with all fluoroquinolones. A growing pool of fluoroquinolone-susceptible isolates that are only a single step away from becoming fully resistant to all fluoroquinolones threatens the longevity of fluoroquinolones as a treatment option. Davies *et al* investigated the emergence of fluoroquinolone resistance by monitoring the prevalence of first-step mutations in levofloxacin-susceptible isolates of *S. pneumoniae*[4]. They reported an increase in the prevalence of first-step mutations in levofloxacin-susceptible isolates of *S. pneumoniae* from isolates collected in 1992-1996 compared to those collected in 1999-2000 [4].

Furthermore, the role of efflux in contributing to fluoroquinolone resistance has yet to be elucidated. It has been suggested that an active efflux mechanism allows an organism to survive and replicate in the presence of the potentially lethal fluoroquinolone until a more stable resistance mechanism, such as a *parC* mutation, is acquired. Following from this belief, the possibility of isolates with active efflux mechanisms being hypermutators has been suggested and is of great concern regarding the potential emergence of resistance[64].

As evidence of the importance of NWT *S. pneumoniae* isolates is increasing, current methods of detecting fluoroquinolone resistance continue to fail to identify susceptible isolates exhibiting mechanisms contributing to resistance. Though it is well documented that double mutations in *parC* and *gyrA* lead to clinically relevant levels of fluoroquinolone resistance in *S. pneumoniae*, there remains a lack of understanding of the contributions of single-step mutations and efflux. Reports on fluoroquinolone efflux in *S. pneumoniae* vary immensely. These discrepancies are largely due to the use of different methods in determining efflux.

Hypothesis:

Current phenotypic methods based on MICs are inappropriate tools for detecting the presence of fluoroquinolone resistance associated mechanisms in NWT isolates of *S. pneumoniae*.

Objectives:

1. To assess the prevalence of NWT fluoroquinolone susceptible isolates with levofloxacin MICs of 1 μg/mL in order to ascertain a levofloxacin susceptibility breakpoint that more accurately describes the wild-type population than does the current CLSI breakpoint of ≤ 2 μg/mL.

2. To test the validity of the reserpine agar dilution assay as an appropriate method for screening for fluoroquinolone efflux by comparing accumulation of various phenotypes of *S. pneumoniae*.

2. Materials and Methods

2.1. Bacterial Isolation and Identification

Most isolates used for the studies outlined in this thesis were collected as part of an ongoing Canadian Respiratory Organism Susceptibility Study. Beginning in October 1997, 25 medical centres from 9 of the 10 Canadian provinces were asked to collect and submit 100 consecutive respiratory isolates of *S. pneumoniae* and 50 consecutive isolates of bacteremic *S. pneumoniae*. All organisms were deemed significant and identified as *S. pneumoniae* according to each laboratory's standard procedures. Each isolate was subcultured on 5% sheep blood agar (SBA) and incubated for 24 hours at 35°C in 5 to 10% CO_2. Amies semisolid transport medium with charcoal (Difco Laboratories, Detroit, Michigan) was used to transport the isolates to the Health Sciences Centre where they were subcultured on 5% SBA and incubated for 18-24 hours in 10% CO_2 and reconfirmed to be *S. pneumoniae* via bile solubility and optochin disk testing. Each isolate was stocked in skim milk and stored at -80°C. Isolates collected as part of similar surveillance studies that pre-dated CROSS (1995-1997) were also used.

2.2. Sample Selection

2.2.1. 1997-2003 Study of Fluoroquinolone Susceptible Isolates

All isolates of *S. pneumoniae* used in this study were identified as ciprofloxacin-susceptible (ciprofloxacin MIC ≤ 2 μg/mL) and had a levofloxacin MIC of 1μg/mL. A total of 776 isolates were identified that fit these criteria, with 111 isolates from the cohort collected prior to the introduction of respiratory fluoroquinolones for clinical use in Canada (1995-1997) and 665 isolates collected after seven years of use (2003).

2.2.2. Ciprofloxacin Efflux Study Isolates

All *S. pneumoniae* isolates with a ciprofloxacin MIC ≥ 4 μg/mL collected throughout the course of CROSS were previously characterized as follows: QRDRs of *parC* and *gyrA* were sequenced, the presence of the putative efflux pump *pmrA* was determined by PCR, and the presence of an active efflux mechanism was assessed via reserpine agar dilution[65]. All isolates with ciprofloxacin MICs of 4 and 8 μg/mL that were identified as efflux positive via the agar dilution assay, including 8 isolates with wild-type *gyrA* and *parC* QRDRs and 5 isolates with a single *parC* QRDR mutation, were further characterized by full gene sequencing of *parC*, *gyrA* and *parE*. In addition, 3 wild-type isolates (ciprofloxacin MIC = 1 μg/mL) and 9 low-level ciprofloxacin resistant (MIC = 4 or 8 μg/mL) reserpine agar dilution negative isolates with resistance associated QRDR mutations in *parC* were characterized for comparison. At least three isolates of each phenotype/genotype group were used for the ciprofloxacin accumulation assay.

2.3. Susceptibility testing – Broth Microdilution

In accordance with the 2003 NCCLS M7-A6 broth microdilution standard, 96-well microtiter plates containing doubling dilutions of each antimicrobial agent in 100 μL of cation-adjusted Mueller-Hinton broth with 2% to 5% vol/vol lysed horse blood were prepared in house. Antimicrobials were prepared from laboratory grade powders obtained from their respective manufacturers. They were reconstituted and stored according to CLSI guidelines. The fluoroquinolones tested for each isolate were ciprofloxacin (Bayer Canada Inc.), gatifloxacin (Bristol-Myers Squibb Canada), gemifloxacin (GlaxoSmith-Kline), levofloxacin (Janssen-Ortho Inc.) and moxifloxacin (Bayer Canada Inc.). Control

strains used for confirmation of antimicrobial activity included *S. pneumoniae* ATCC® 49619, *S. aureus* ATCC® 29213 and *Pseudomonas aeruginosa* ATCC® 27853.

Following two subcultures on SBA from frozen stock, bacterial suspensions in sterile water were prepared and adjusted to a 0.5 McFarland Standard (approximately 1-2 x 10^8 CFU/mL). Microtitre plates were inoculated with a final bacterial concentration of 5 x 10^5 CFU/mL and incubated at 35°C in ambient air for 24 hrs. Confirmation of inocula was obtained by performing colony counts. The lowest well exhibiting no visible growth was recorded as the MIC. MICs for ciprofloxacin resistant isolates were performed in at least triplicate on separate days.

2.4. Lysate Preparation

For each isolate of *S. pneumoniae*, a small amount of bacteria from an overnight culture grown on SBA was emulsified in lysis solution as described by Ubukata *et al.* Using a Perkin-Elmer GeneAmp® PCR System 9700 the cell suspension was lysed by incubation at 60°C for 10 minutes followed by incubation at 94°C for 5 minutes. Lysates were used as genomic DNA templates for the PCR reactions described in section 2.5.

2.5. PCR Amplification and Purification

All PCR amplification reactions were performed using the Perkin-Elmer GeneAmp® PCR System 9700. All primers used for PCR amplification of the various genes are listed in Table 2.1.

Table 2.1 **Primers used for PCR amplification of *gyrA* and *parC* QRDRs, and full gene products of *gyrA, parC, parE* and *pmrA*.**

Primer	Sequence (5'-3')	Nucleotide Position	Reference
GyrA fwd	TAAAAAACTTTGTCACGAATATGCC	130-105 bp upstream of start	67
GyrA rev-QRDR	AACGATACGCTCACGACCAGT	750-771	67
GyrA rev-Full	GAGACATTATGCTTCACCTTCTG	2453-2475	This study
ParC fwd	AAACCTACTCTACATTCTTTGAAAGGAG	134-106bp upstream of start	67
ParC rev-QRDR	CAGTTGGGTGGTCAATCATGTAAA	571-594	67
ParC rev-Full	CCACTCCTTATTCTAAAAACC	2579-2599	This study
ParE fwd	CCAGATGGAATCGAACCC	191-175 bp upstream of start	This study
ParE rev-Full	CCTTTCAAAGAATGTAGAGTAGG	2234-2256	This study
Pmr5 Fwd[1]	GGATCCGATATCAGCCAGGTTCTTGATA	80-64 bp upstream of start	66
Pmr3 rev	GGTACCAAGCTTCTAGATTTCCTTTACTTTTA	1181-1202	66

[1]Restriction sites are underlined.

Amplification of the *pmrA* gene was performed using primers and conditions previously described by Nickerson *et al*[66].

The QRDRs of *parC* and *gyrA* were amplified using primers and conditions described by Morrissey *et al*[67].

The amplification of the *gyrA, parC* and *parE* genes in full was accomplished using 4 primers previously described by Morrissey and George[67] and 4 newly designed primers listed in Table 2.1. Each amplification reaction contained 5 μL of 15 mM $MgCl_2$-10X PCR buffer, 1 mM of dNTPs, 10 μM of each of the forward and reverse primers, 1 mM $MgCl_2$, 2.5 U of Taq DNA polymerase, 5 μL of DNA template, and the addition of sterile water to a final volume of 50 μL. Thermocycler conditions for *parC* and *parE* consisted of an initial denaturing step of 5 minutes at 94°C followed by 30 cycles of 94 °C for 30 seconds, 50 °C for 1 minute, and 72 °C for 3 minutes and a final extension for 7 minutes at 72°C. Cycling conditions for *gyrA* varied slight with 30 cycles of 94 °C for 30 seconds, 57 °C for 45 seconds and 72 °C for 2 minutes and 15 seconds. Initial denaturation and final extension remained the 94°C for 5 minutes and 72°C for 7 minutes respectively.

After amplification, confirmation of DNA fragments was obtained by agarose gel electrophoresis. Agarose gels were made with 0.5X Tris-Borate-EDTA and containing ethidium bromide. One percent agarose gels were used for analysis of full gene products of *gyrA, parC, parE* and *pmrA,* while 2% gels were used for QRDR fragments.

2.6. Sequencing of *parC* and *gyrA* QRDRs and Full Gene Products of *parC, gyrA* and *parE*

Microcon® YM100 centrifugal filter units (Millipore, Bedford, MA) were used to purify PCR products according to the manufacturer's instructions. Elution of the purified PCR products from the columns was carried out using Tris (10mM)-EDTA (1mM) pH 8.0 buffer. DNA quantitation was performed using the nucleic acid mode of an Ultrospec 2100 pro (Amersham Biosciences, Baie d'Urfe, QC), which calculates the DNA concentration of the sample based on the knowledge that at 260 nm, DNA with an optical density (OD) of 1 is at a concentration of 50 μg/mL in a 10 mm pathlength cell.

Sequencing reactions were prepared with the ABI PRISM® BigDye Terminator Cycle Sequencing Ready Reaction Kit using conditions described by Zhanel *et al*[65]. Sequencing in both the forward and reverse directions was accomplished with the primers listed in Table 2.2. The primers used to sequence the QRDRs of *gyrA* and *parC* have been previously described [67]. The primers designed for this study to sequence the full-length genes of *gyrA*, *parC*, and *parE* were created to be 18 to 21 bp in length and generate overlapping sequences. Cycle sequencing was performed on the Perkin-Elmer Gene Amp® PCR System 9700.

As recommended in the ABI PRISM® BigDye Terminator Cycle Sequencing Ready Reaction Kit information enclosure (Applied Biosystems, Foster City, CA), sequencing products were purified with sodium acetate and ethanol[65]. Purified sequencing products were reconstituted in 15 μL formamide for analysis on the ABI 3100 Genetic Analyzers (Applied Biosystems, Foster City, CA).

Table 2.2 Primers used for sequencing amplification of PCR products of *parC*, *parE* and *gyrA*.

Primer	Sequence (5'-3')	Nucleotide Position	Reference
QRDRs of *gyrA* and *parC*			
GyrA SF	CGTTTTAGTGGTTTAGAGGC	85-66 bp upstream of start	67
GyrA SR	GACCAACTTCACTGCATC	567-585	67
ParC SF	CGCCCTAGATACTGTGTGA	98-80 bp upstream of start	67
ParC SR	AAATCCCAGTCGAACCAT	493-510	67
gyrA*, *parC*, and *parE			
GyrA SF2	GGTAAATACCACCCACACG	217-236	This study
GyrA SF3	GATGCAGTGAAGTTGGTC	567-585	This study
GyrA SF4	GGGTATCACAGCAGTACG	852-868	This study
GyrA SF5	CGCTAGACCATATCGACG	1148-1165	This study
GyrA SF6	GCCGTACAGAGTTGATGG	1439-1460	This study
GyrA SF7	GCTACCAGTAGTCAATCTC	1740-1758	This study
GyrA SF8	CGTATCGCCACTGGTGTG	2041-2058	This study
GyrA SR1	GCCACCGCAACCGTTG	2420-2405	This study
GyrA SR2	CACACCAGTGGCGATACG	2041-2058	This study
GyrA SR3	GAGATTGACTACTGGTAGC	1740-1758	This study
GyrA SR4	CCATCAACTCTGTACGGC	1439-1460	This study
GyrA SR5	CGTCGATATGGTCTAGCG	1148-1165	This study
GyrA SR6	CGTACTGCTGTGATACCC	852-868	This study
ParC SF2	CTGAGGCACGTTTGTCTG	356-373	This study
ParC SF3	CGTGATGAAATCAAGAAAGC	664-683	This study
ParC SF4	GCTAATACTGAGCTTGTTC	916-934	This study
ParC SF5	GGAAAACCTCAAAGTTAGC	1206-1224	This study
ParC SF6	GGCAGGTTACATCAAGCG	1524-1541	This study
ParC SF7	CGACAACCTACTTTGCAG	1805-1822	This study
ParC SF8	GTCCTTCTACCTCTTGACC	2109-2127	This study
ParC SR1	CCAGCTCTTAGAACTTATTC	2502-2483	This study
ParC SR2	GGTCAAGAGGTAGAAGGAC	2109-2127	This study
ParC SR3	CTGCAAAGTAGGTTGTCG	1805-1822	This study
ParC SR4	CGCTTGATGTAACCTGCC	1524-1541	This study
ParC SR5	GCTAACTTTGAGGTTTTCC	1206-1224	This study
ParC SR6	GAACAAGCTCAGTATTAGC	916-934	This study
ParC SR7	GCTTTCTTGATTTCATCACG	664-683	This study
ParE SF1	CTGCTGAAATTGTCACATC	74-92 bp upstream of start	This study
ParE SF2	GACGGTAGTCTAACGGTTC	211-229	This study
ParE SF3	CACCAAAGTTACTTTTATGC	513-532	This study
ParE SF4	CAGATAACATTCTATCCTTTG	803-823	This study
ParE SF5	GGAATTAGCTTCTAACCTC	1116-1132	This study
ParE SF6	CCAAGATGGCGGATATCC	1394-1411	This study

ParE SF7	GGTAAAGGCGCTACCCTC	1714-1731	This study
ParE SR1	CTGTCGCTTCTTCTAGCG	1919-1936	This study
ParE SR2	GAGGGTAGCGCCTTTACC	1714-1731	This study
ParE SR3	GGATATCCGCCATCTTGG	1394-1411	This study
ParE SR4	GAGGTTAGAAGCTAATTCC	1116-1132	This study
ParE SR5	CAAAGGATAGAATGTTATCTG	803-823	This study
ParE SR6	GCATAAAAGTAACTTTGGTG	513-532	This study
ParE SR7	GAACCGTTAGACTACCGTC	211-229	This study

Sequence analysis was conducted on the ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) in accordance with the manufacturer's instructions. Forward and reverse sequences for each isolate were aligned and compared to the published sequence of *S. pneumoniae* R6 using ABI PRISM® SeqScape® Software (Applied Biosystems, Foster City, CA).

2.7. Reserpine Agar Dilution

The agar dilution assay was modified from the method described by CLSI to incorporate reserpine as a potential efflux pump inhibitor. The protocols were provided by Dr. D. Bast (Mt. Sinai Hospital, Toronto, ON)[49].

Two sets of Mueller-Hinton agar plates with 5% sheep's blood containing doubling dilutions of ciprofloxacin were prepared in duplicate with one set containing 10 μg/mL reserpine. Ciprofloxacin concentration ranges were determined based on the MIC ranges (as previously determined by microbroth dilution) of the isolates being tested. The concentrations ranged from 4 doubling dilutions below the lowest expected MIC to 2 dilutions greater that the highest expected MIC to account for both differences in MICs due to variable growth in broth compared to solid medium and for the potential 8-fold decrease in MIC for possible efflux positive isolates.

Each isolate was run in at least duplicate along with control strains *S. pneumoniae* ATCC® 49619, *Enterococcus faecalis* ATCC® 29212, *P. aeruginosa* ATCC® 27853 and *S. aureus* ATCC® 29213. Bacterial suspensions of each isolate were adjusted to a 0.5 McFarland standard in Mueller-Hinton broth. Suspensions were diluted 1:2 in broth. 0.5 mL of each dilution was loaded into the wells of a Steer's replicator such that the

final inoculum delivered by the replicator's pins was approximately 10^4 CFU. The streer's replicator was used to inoculate the plates in order of increasing ciprofloxacin concentration. SBA was inoculated before and after the ciprofloxacin-containing plates as a growth control and to ensure that there was no contamination or significant antimicrobial carry-over during inoculation. Plates were incubated at 35°C for 20-24 hours in CO_2. The duplicate plates were incubated at 35°C in ambient air for optimal growth conditions of control organisms *E. faecalis, P. aeruginosa* and *S. aureus.*

Ciprofloxacin MICs in the presence and absence of reserpine were recorded after incubation for each isolate. Isolates exhibiting a 4-fold or greater reduction in MIC in the presence of reserpine were considered positive for reserpine-sensitive efflux [49].
All isolates were tested in at least duplicate on separate days to ensure reproducibility.

2.8. Fluoroquinolone Accumulation

The fluorescence accumulation method described by Piddock and Johnson was used with slight modifications to measure the amount of ciprofloxacin accumulated by each strain[46]. For each strain a 0.5 McFarland was prepared in normal saline and diluted 1 in 2. Three-hundred and fifty µL of the diluted culture was added to 75 mL of brain heart infusion (BHI) broth which was incubated at 37°C in CO_2 for no more than 10 hours. Twenty-five mL of the starter culture was added 175 mL BHI and incubated at 37°C in CO_2 until growth reached an OD of A_{660}=0.6-0.7 (approximately 4 hours).

Cells were harvested by centrifugation at 10,000 x *g* and washed once in cold 0.1 M sodium phosphate buffer pH 7.0. After further centrifugation, cells were resuspended in 0.1 M sodium phosphate buffer to an A_{660}=20. The culture was divided in two and

reserpine was added to one half of the divided culture to a final concentration of 20 μg/mL. At this point a 100 μL sample of each culture was taken, diluted to 10^{-8}, plated on Mueller-Hinton blood agar and incubated at 37°C in CO_2 overnight to obtain colony counts. The two cultures were allowed to equilibrate at 37°C in CO_2 for 10 minutes.

After incubation a 0.5 mL sample of each culture was removed and placed in 1 mL ice cold sodium phosphate buffer prior to adding ciprofloxacin, these samples were later considered T=0 and were used to blank the fluorometer. Ciprofloxacin was then added to each culture to a final concentration of 10 μg/mL. Samples of 0.5 mL were taken from each culture every minute from T=1 min to T=5 min as well as at T=8 min and T=10 min and placed in cold sodium phosphate buffer and kept on ice. Samples were centrifuged at 7300rpm for 5 minutes. The supernatant was removed and cells were washed in ice-cold sodium phosphate buffer and centrifuged for another 5 minutes. Once again, the supernatant was removed and the cells were resuspended in 0.1 M glycine-HCl pH 3.0. Cells were allowed to lyse in glycine-HCl for at least two hours at room temperature in the dark.

The lysed samples were centrifuged a final time for 10 minutes at 7300rpm. The supernatant was transferred to fresh 1.5 mL tubes and pellets were placed in the fumehood to dry. Samples were diluted 1:4 in glycine-HCl for reading on the LS 55 Luminescence Spectrometer (Perkin Elmer Ltd., Buckinghamshire UK). Sample concentrations were calculated using FL Winlab software (Perkin Elmer, Buckinghamshire UK) by comparison to a standard curve of ciprofloxacin prepared in glycine-HCl. Dry weight of cells was measured. Final results were reported as both ng ciprofloxacin per mg bacteria and ng ciprofloxacin per 10^9 cells.

2.9. Statistical Analysis

The statistical significance between groups of fluoroquinolone-susceptible isolates was determined by two-tailed Fisher's Exact Test using GraphPad Software (San Diego, CA).

3. Results

3.1. Identifying NWT Levofloxacin Susceptible Isolates

One-hundred and eleven isolates with ciprofloxacin MICs of ≤ 2 μg/mL and levofloxacin MICs of 1 μg/mL were collected between 1995 and 1997 and 665 were collected in 2003. The presence of fluoroquinolone resistance-associated mechanisms was assessed for each isolate by sequencing the QRDRs of *gyrA* and *parC*, by performing PCR for the *pmrA* gene and by conducting the reserpine agar dilution assay.

None of the isolates tested exhibited a 4-fold or greater decrease in ciprofloxacin MIC in the presence of reserpine. Two isolates collected in 2003 displayed large deletions of approximately 370bp in *pmrA* compared to R6 (figure 3.1).

All QRDR amino acid substitutions are listed in Table 3.1. Table 3.2 highlights those mutations that have previously been shown to be associated with fluoroquinolone resistance. The prevalence of isolates that had any mutations in the QRDRs of *parC* and *gyrA* did not increase from 1997-2003. Similarly, there was no significant increase in prevalence of isolates possessing a fluoroquinolone resistance associated mutation (P=0.34).

Isolates were subsequently divided into groups based on ciprofloxacin MIC to assess whether a trend was evolving amongst isolates with a ciprofloxacin MIC of 2 mg/L and levofloxacin MIC of 1 mg/L in comparison to isolates with a ciprofloxacin MIC of 1mg/L and a levofloxacin MIC of 1 mg/L. The percentage of isolates harbouring resistance-associated mutations from the cohort with a ciprofloxacin MIC of 2 mg/L increased from 2.6% (1995-1997) to 10.8% (2003) over the seven-year period (p=0.12).

Figure 3.1 **Amplification products of *pmrA* on a 1% agarose visualized with ethidium bromide. Lanes 1- clinical isolate 37391. Lane 2 – clinical isolate 38968. Lane 3 – clinical isolate 48431. Lane 4 – clinical isolate 48434. Lane 5 – Lab strain R6. Lane 6 – Lab strain Tigr4.**

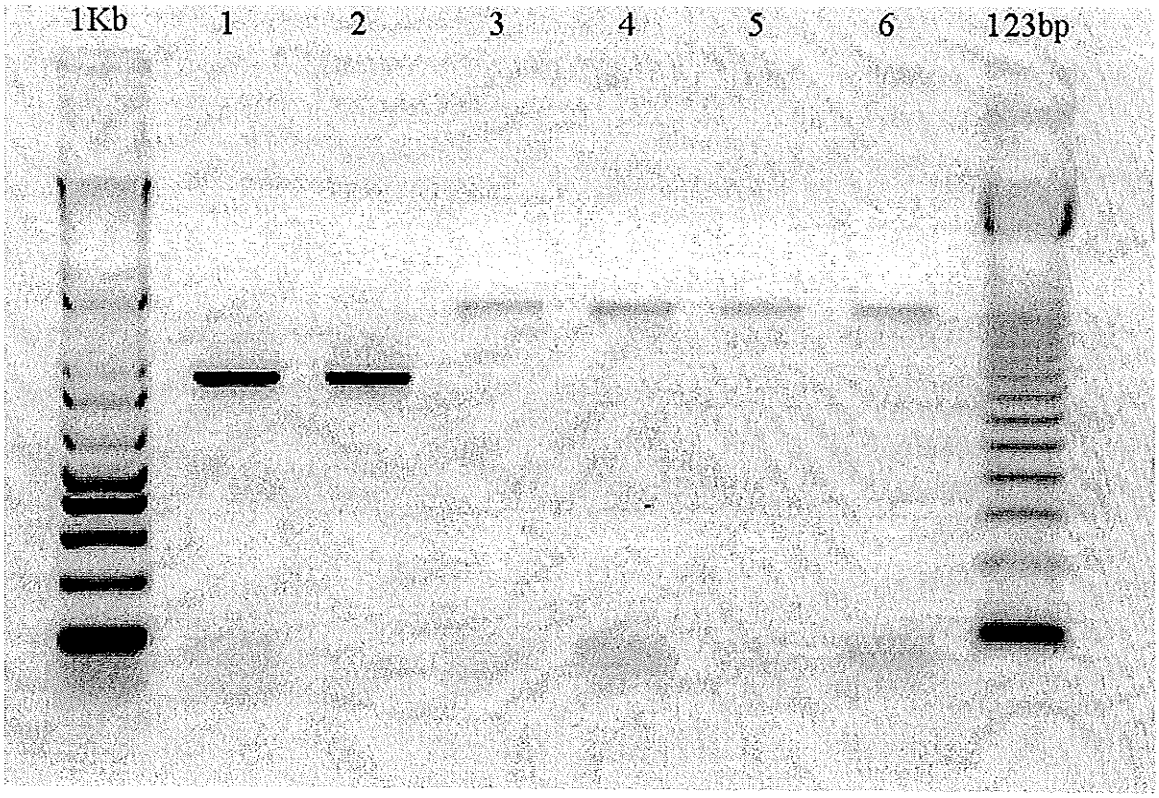

1Kb
1
2
3
4
5
6
123bp

Table 3.1 All amino acid substitutions observed in fluoroquinolone-susceptible *S. pneumoniae* (isolates with levofloxacin MICs of 1 mg/L) between 1995-1997 and in 2003.

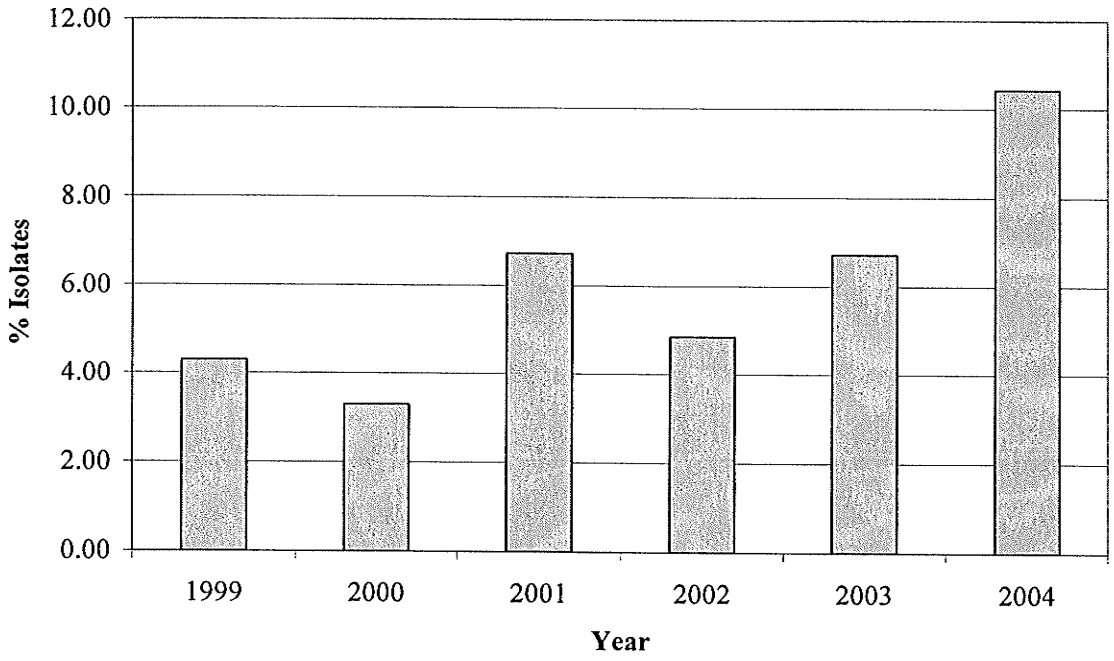
ParC Substitution(s)	GyrA Substitution	No. of isolates in 1995-1997 (N=111)	No. of isolates in 2003 (N=665)
Ser52Asn	None	0	1
Ser52Gly, Lys137Asn	None	1	9
Ser79Phe	None	1	7
Ser79Phe	Ser81Phe	0	1
Ser79Phe, Lys137Asn	None	0	2
Ser79Tyr	None	0	3
Asp83Asn	None	0	1
Arg95Cys	None	1	0
Tyr129Ser	None	0	3
Glu135Asp	None	0	1
Lys137Asn	None	27	132
Lys137Asn	Tyr98Asp	0	1
Total no. of isolates with mutations		30	161
Percent isolates with mutations		**27.0**	**24.2**

Table 3.2 Number of fluoroquinolone-susceptible *S. pneumoniae* (isolates with levofloxacin MICs of 1 mg/L) between 1995-1997 and in 2003 containing known fluoroquinolone resistance-associated QRDR amino acid substitutions.

ParC	GyrA	No. of isolates in 1995-1997 (N=111)	No. of isolates in 2003 (N=665)
Ser79Phe	None	1	9
Ser79Phe	Ser81Phe	0	1
Ser79Tyr	None	0	3
Asp83Asn	None	0	1
Total no. isolates with mutations		1	14
Percent isolates with mutations		**0.9**	**2.1**
Percent isolates exhibiting efflux		**0.0**	**0.0**

Additionally, as seen in figure3.2, we observed a significant increase over time in the prevalence of isolates that had both a ciprofloxacin MIC of 2 mg/L and a levofloxacin MIC of 1 mg/L from 3.9% to 6.5% (p=0.0021).

Figure 3.2 **Percentage of isolates collected from CROSS with ciprofloxacin MICs of 2 µg/mL and levofloxacin MICs of 1 µg/mL by year.**


12.00
10.00
8.00
6.00
4.00
2.00
0.00
% Isolates
1999
2000
2001
2002
2003
2004
Year

3.2. Investigating Low-Level Ciprofloxacin Resistant Isolates

A select group of isolates was characterized by full gene sequencing prior to performing the ciprofloxacin fluorescence accumulation assay. The isolates were divided into four phenotype/genotype groups based on reserpine agar dilution results and QRDR sequencing. The groups consisted of 3 wild type isolates, 8 reserpine sensitive isolates with wild-type ParC and GyrA QRDRs, 5 reserpine-sensitive isolates with resistance associated QRDR amino acid substitutions in ParC and wild type GyrA QRDRs, and 9 isolates with resistance-associated ParC QRDR amino acid substitutions, negative reserpine agar dilution results and wild-type GyrA QRDRs. As seen in Table 3.3 all isolates including wild-type isolates possessed ParC and GyrA amino acid substitutions compared to lab strain R6. The wild-type isolates were however identical to each other in their ParC, GyrA and ParE subunits. A large degree of heterogeneity was seen amongst the ciprofloxacin resistant isolates, which all possessed at least 1, and up to as many as 8 amino acid substitutions in ParC, GyrA and/or ParE compared to the wild-type isolates.

A subset of the isolates characterized above, including at least 3 representatives of each phenotype, was selected for evaluating ciprofloxacin accumulation in the presence and absence of reserpine. Ciprofloxacin accumulation was observed over a 10 minute period. Similar to previous studies of fluoroquinolone accumulation, all isolates appeared to rapidly reach a steady state before 5 minutes. Data was reported as both ng of ciprofloxacin per mg bacteria and as ng of ciprofloxacin per 10^9 cells. Examples of fluorescence accumulation in representative isolates of each phenotype are shown in figures 3.3 through 3.6. Addition of reserpine did not result in an overall increase in

Table 3.3 **Amino acid substitutions found in isolates characterized by full gene sequencing.**

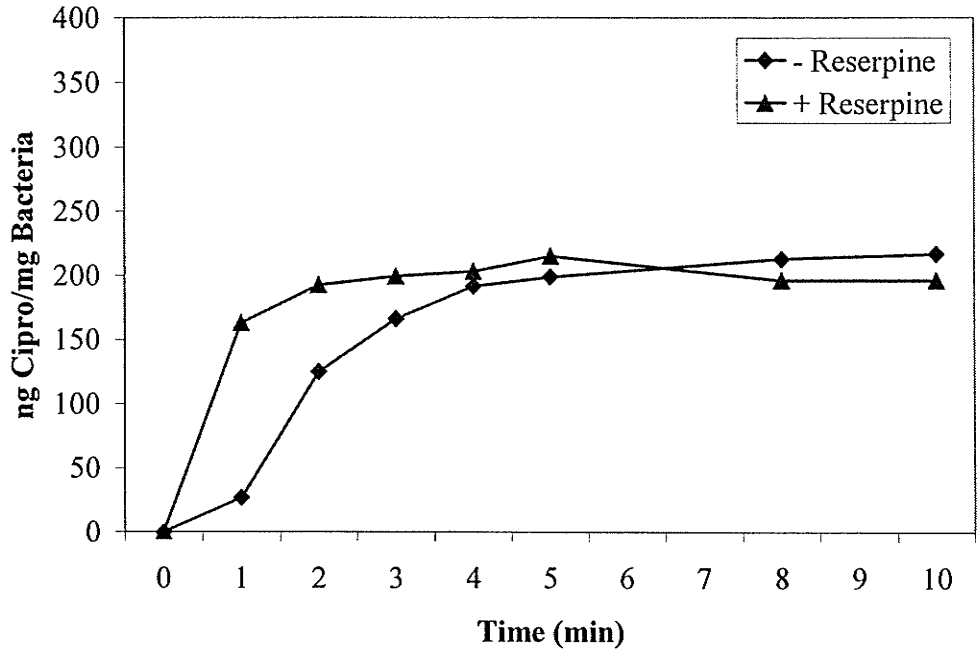
Strain #	Cipro MIC	GyrA[1]	ParC	ParE
Wild-Type Isolates				
2587	**1**	Val489Ile	Asp822Tyr	None
2663	**1**	Val489Ile	Asp822Tyr	None
2670	**1**	Val489Ile	Asp822Tyr	None
Reserpine Agar Dilution Positive Isolates with Wild-Type ParC and GyrA QRDRs				
1282	**8**	Val489Ile	Lys137Asn, Val608Ala	Il460Val, Glu474Lys
15017	**4**	Val489Ile, Ala653Thr	Ala394Thr	Gly34Arg, Ile460Val
16072	**4**	Val489Ile, Ala653Thr	His373Arg, Glu589Ala, Lys437Asn, Val608Ala	Ile460Val
29012	**4**	Val486Ile	Ala189Val, Thr493Ile, Val608Ala	Ile162Val
29262	**4**	Ala405Asp, Arg510Lys, Asp519Ala, Ser594Asn	Lys137Asn, Lys445Arg, Ser553Thr, Val608Ala	ND
30890	**4**	Val486Ile	Lys137Asn, Arg569Cys	Ser200Leu
44171	**4**	Val489Ile, Ala653Thr	Ala450Val, Glu589Ala, Val608Ala	Ile460Val
55073	**4**	Val489Ile, Ala653Thr	ND	ND
Reserpine Agar Dilution Positive Isolates with ParC QRDR Mutations				
10277	**8**	Val489Ile, Ala653Thr	**Ser79Phe**, Ala450Val	Ile460Val
12873	**8**	Val489Ile, Ala653Thr	**Ser79Phe**, Ala450Val	Ile460Val
17913	**8**	Ser237Leu, Val486Ile	**Asp83Gly**, Asp294Leu, Ile453Tyr, Val608Ser	Asp217Asn
19120	**8**	Val486Ile, Ser778Leu	**Asp83Asn**	Ile460Val
51597	**4**	Val489Ile, Ala653Thr	**Ser79Phe**	Val355Ile, Ile460Val
Reserpine Agar Dilution Negative Isolates with ParC QRDR Mutations				
4610	**4**	Val489Ile	**Ser79Phe,** Ala450Val, Glu589Ala, Val608Ala	Ile460Val
11434	**4**	Val489Ile, Ala653Thr	**Ser79Phe**, Lys137Asn	Ser132Asn, Leu290Phe, Ala326Val, Ile460Val
12291	**4**	Val489Ile, Ala653Thr	**Ser79Tyr**, Ala450Val, Glu589Ala, Val608Ala	Ile460Val
12883	**4**	Val489Ile, Ala653Thr	**Ser79Tyr**, Lys137Asn	ND
14744	**4**	Val489Ile, Ala653Thr	**Ser79Phe**, Val608Ala	Ile162Val, Thr216Ser
14769	**8**	Val489Ile	**Ser79Phe,** Lys137Asn, Val608Ala	Ile460Val

20336	**8**	Val489Ile	**Asp83Ala**, Lys137Asn, Val608Ala	None
22360	**8**	Val489Ile	**Asp83Ala**, Lys137Asn, Val608Ala	Ile460Val
22627	**8**	Val768Phe	**Asp83Gly**, Ala189Val, Val608Ala	Pro166Leu, Thr212Ala, Ile460Val

[1] Resistance-associated QRDR amino acid substitutions are listed in bold.

Figure 3.3 **Ciprofloxacin accumulation in wild-type isolate 2587 in the presence and absence of reserpine. A) Results presented as ng of ciprofloxacin per mg bacteria. B) Results presented as ng of ciprofloxacin per 10^9 cells.**

A)


400
350
300
250
200
150
100
50
0
ng Cipro/mg Bacteria
- Reserpine
+ Reserpine
0 1 2 3 4 5 6 7 8 9 10
Time (min)

B)

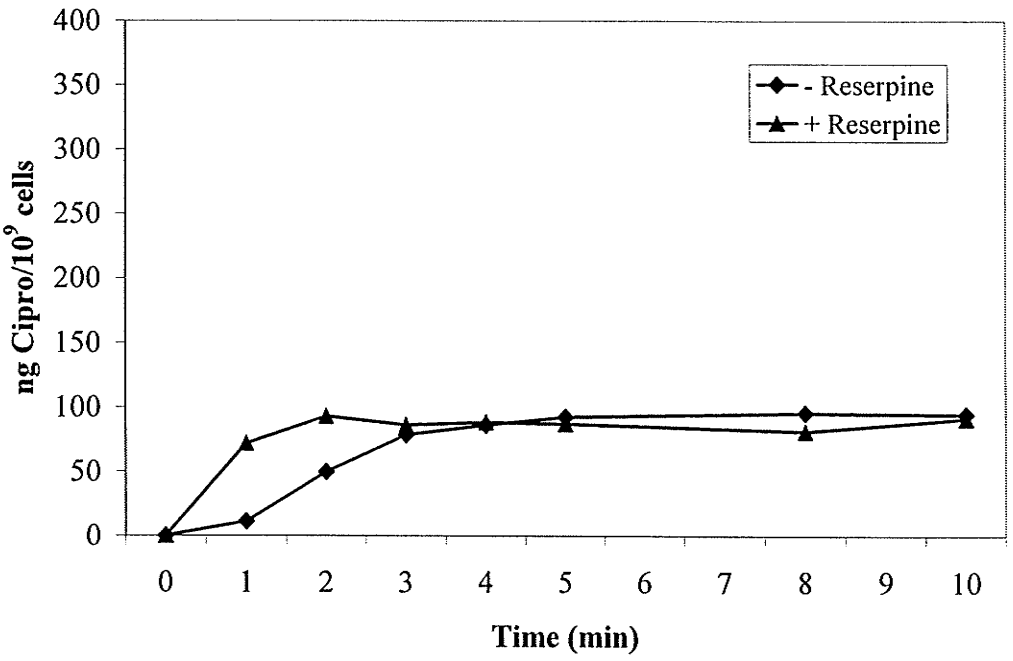
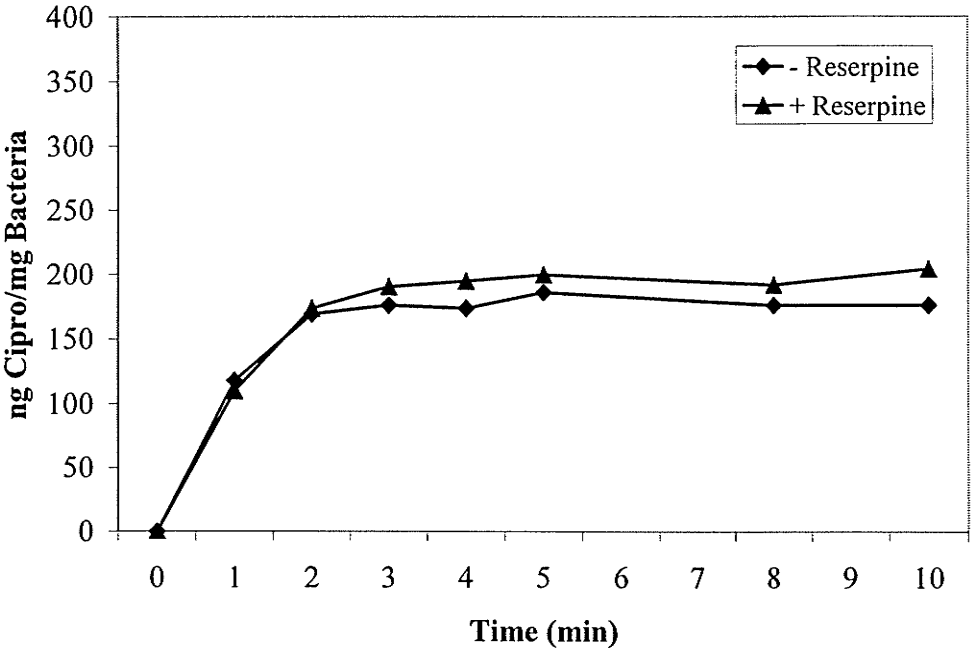

400
350
300
250
200
150
100
50
0
ng Cipro/10^9 cells
- Reserpine
+ Reserpine
0 1 2 3 4 5 6 7 8 9 10
Time (min)

Figure 3.4 **Ciprofloxacin accumulation in reserpine-sensitive isolate 30890 in the presence and absence of reserpine. A) Results presented as ng of ciprofloxacin per mg bacteria. B) Results presented as ng of ciprofloxacin per 10^9 cells.**

A)


400
350
300
250
200
150
100
50
0
ng Cipro/mg Bacteria
- Reserpine
+ Reserpine
0 1 2 3 4 5 6 7 8 9 10
Time (min)

B)

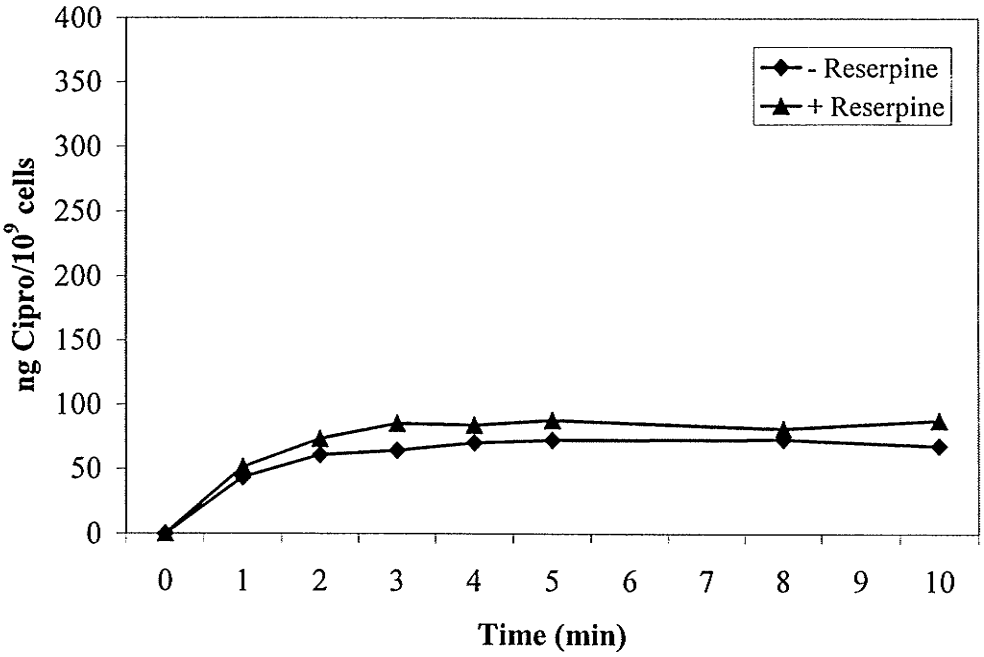
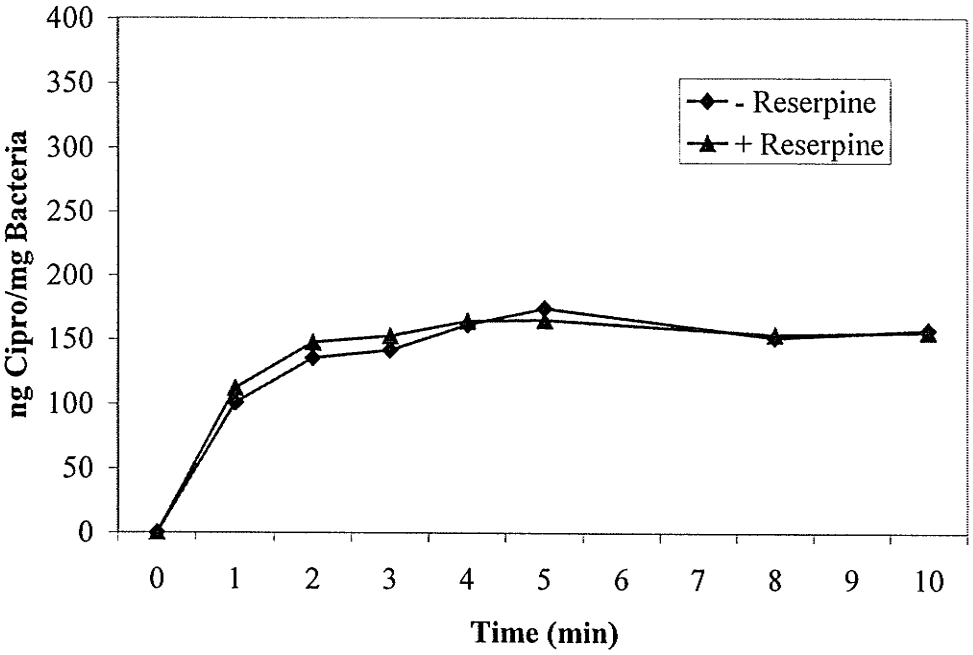

400
350
300
250
200
150
100
50
0
ng Cipro/10^9 cells
- Reserpine
+ Reserpine
0 1 2 3 4 5 6 7 8 9 10
Time (min)

Figure 3.5. **Ciprofloxacin accumulation in ParC mutant strain 14744 in the presence and absence of reserpine. A) Results presented as ng of ciprofloxacin per mg bacteria. B) Results presented as ng of ciprofloxacin per 10^9 cells.**

A)


400
350
300
250
200
150
100
50
0
ng Cipro/mg Bacteria
- Reserpine
+ Reserpine
0 1 2 3 4 5 6 7 8 9 10
Time (min)

B)

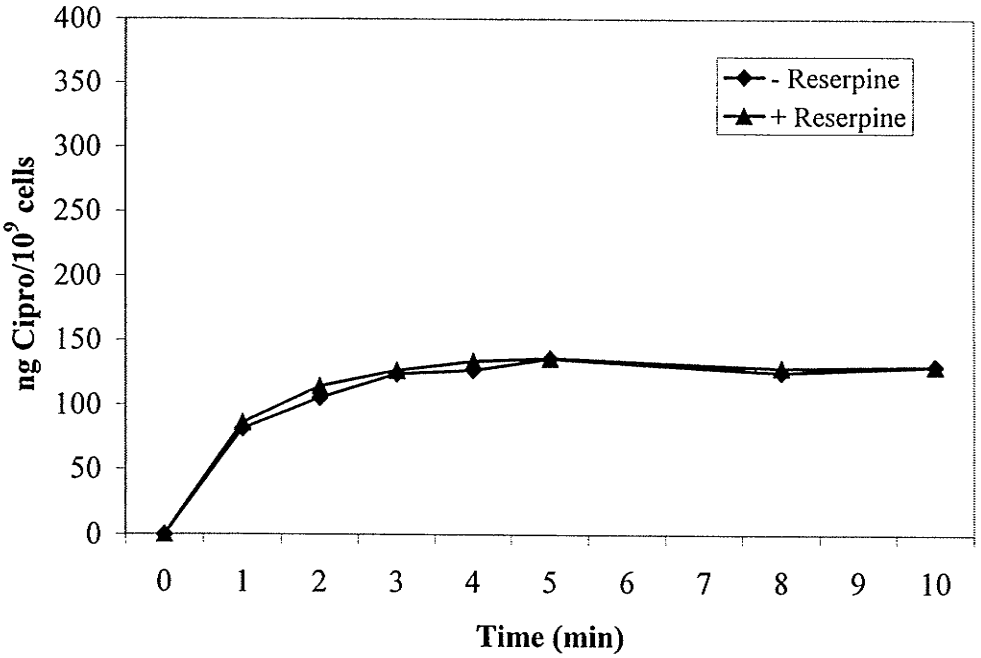
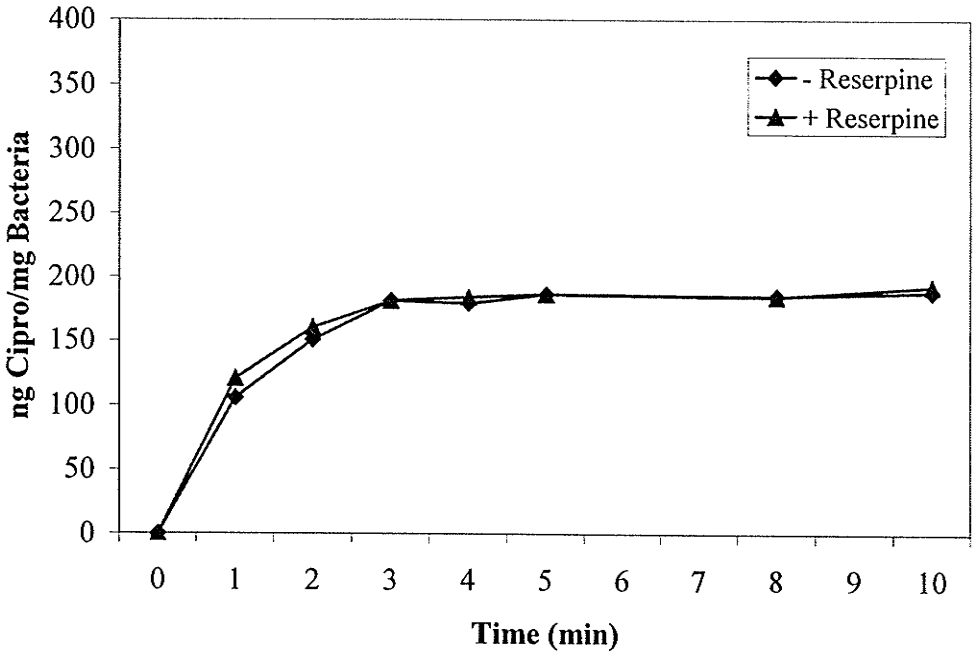
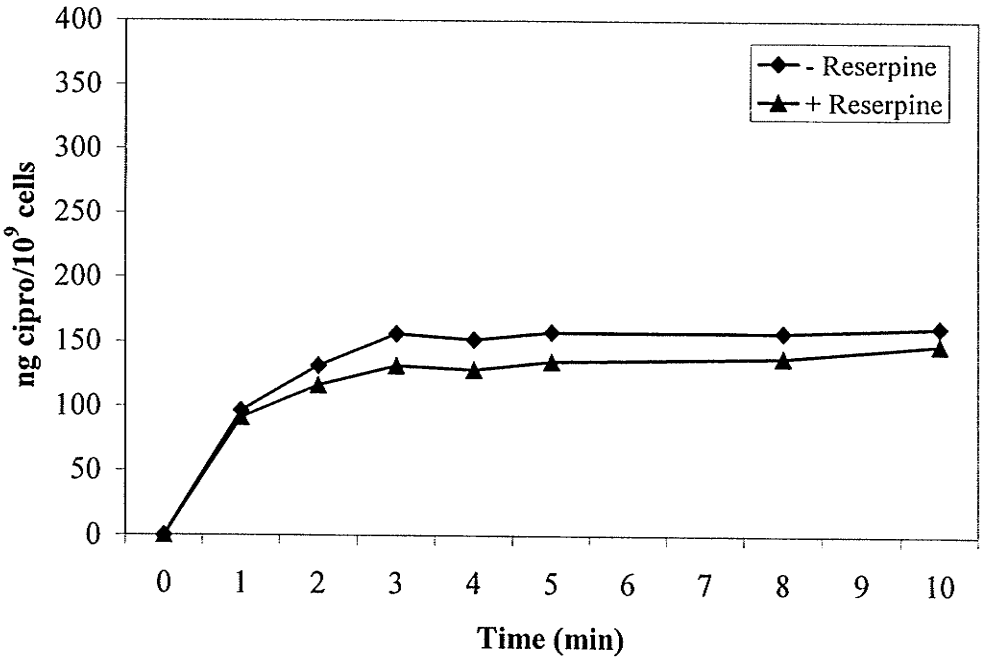

400
350
300
250
200
150
100
50
0
ng Cipro/10^9 cells
- Reserpine
+ Reserpine
0 1 2 3 4 5 6 7 8 9 10
Time (min)

Figure 3.6. **Ciprofloxacin accumulation in reserpine-sensitive ParC mutant Strain 19120 in the presence and absence of reserpine. A) Results presented as ng of ciprofloxacin per mg bacteria. B) Results presented as ng of ciprofloxacin per 10^9 cells.**

A)


400
350
300
250
200
150
100
50
0
ng Cipro/mg Bacteria
- Reserpine
+ Reserpine
0 1 2 3 4 5 6 7 8 9 10
Time (min)

B)


400
350
300
250
200
150
100
50
0
ng cipro/10^9 cells
- Reserpine
+ Reserpine
0 1 2 3 4 5 6 7 8 9 10
Time (min)

ciprofloxacin accumulation in any isolate. Average ciprofloxacin accumulation at T=5 min was also recorded for each phenotype (figure 3.7). No significant difference in total accumulation was observed between the various phenotypes.

Finally, to determine if reserpine-sensitive isolates represented hypermutator strains, the average number of amino acid substitutions per isolate (compared to the wild-type isolates) in each phenotype group was calculated (Table 3.4).

Figure 3.7 **Average ciprofloxacin accumulation in the presence and absence of reserpine among various phenotypes of *S. pneumoniae* 5 minutes after the addition of ciprofloxacin. Phenotype groups: 1 – Isolates with wild type QRDRs, and negative reserpine agar dilution. 2 – Isolates with wild type QRDRs and positive reserpine agar dilution. 3 – Isolates with ParC QRDR mutations and positive reserpine agar dilution. 4 – All isolates with positive reserpine agar dilution. 5 – Isolates with ParC QRDR mutations and negative reserpine agar dilution. 6 – All isolates with ParC QRDR mutations. 7 – All isolates with negative reserpine agar dilution.**

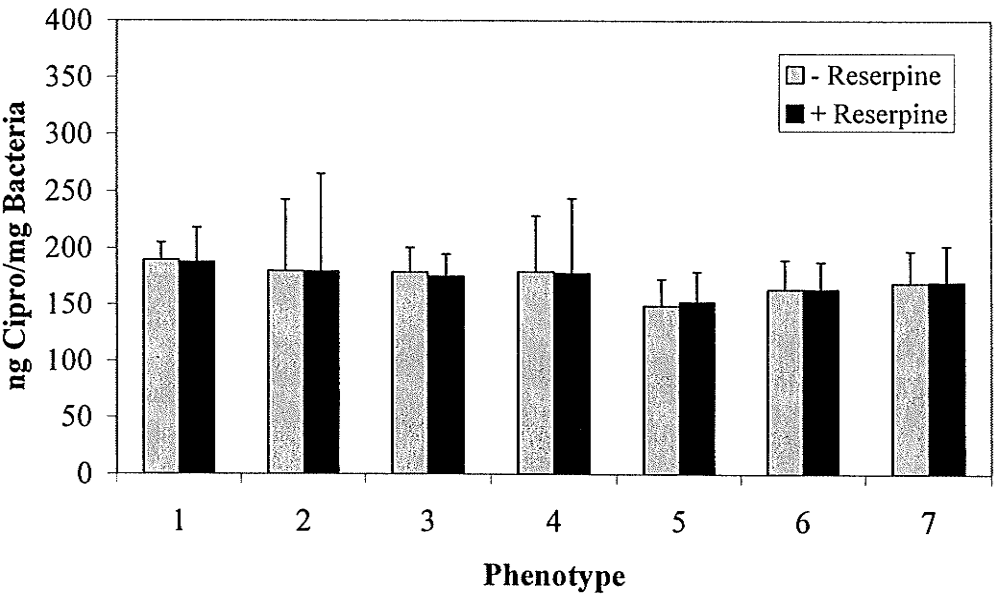

400
350
300
250
200
150
100
50
0
ng Cipro/mg Bacteria
- Reserpine
+ Reserpine
1
2
3
4
5
6
7
Phenotype

Table 3.4 **Average Number of Amino Acid Substitutions in GyrA, ParC and ParE per isolate in each phenotypic group.**

	GyrA	ParC	ParE	Total
Reserpine agar dilution positive, wild type QRDR	1.25	2.71	1.33	4.63
Reserpine agar dilution positive, ParC QRDR mutant	1.40	2.00	1.20	4.60
Reserpine agar dilution negative, ParC QRDR mutant	0.56	2.89	1.63	4.78

4. Discussion

To address the main hypothesis that current phenotypic methods based on MICs are inappropriate tools for detecting the presence of fluoroquinolone resistance associated mechanisms in NWT isolates of *S. pneumoniae,* this thesis focuses on two populations of isolates: fluoroquinolone-susceptible isolates and isolates exhibiting low-level ciprofloxacin resistance.

4.1. Fluoroquinolone-Susceptible Isolates

Fluoroquinolone-susceptible isolates can serve as a baseline for comparison when studying resistant isolates and so it is important to be able to distinguish fluoroquinolone-susceptible wild-type isolates from fluoroquinolone-susceptible NWT isolates. Moreover, NWT isolates present a major risk for treatment failure as these isolates rapidly develop secondary mutations which result in high-level fluoroquinolone resistance. CLSI breakpoints are designed with the ultimate goal of achieving clinical success and do not distinguish between these two groups of susceptible isolates. The first objective of this thesis was to assess the prevalence of NWT fluoroquinolone-susceptible isolates with levofloxacin MICs of 1 μg/mL in order to ascertain a levofloxacin susceptibility breakpoint that more accurately predicts the probability of an isolate being wild-type or NWT. The change in prevalence of NWT isolates in this population of fluoroquinolone-susceptible isolates was also assessed by comparing the prevalence of NWT isolates collected from 1995-1997 (prior to the introduction of levofloxacin) and in 2003 (after seven years of use) to see whether the prevalence of NWT with levofloxacin MICs of 1 μg/mL was increasing as a result of increased respiratory fluoroquinolone use.

4.1.1. Single-Step *parC* Mutations

Previous studies have focused on levofloxacin susceptible isolates with MICs of 2μg/mL and have clearly established that the majority of these isolates are genetically NWT and that the prevalence of *parC* mutations among this population of isolates is increasing. As a follow up to these findings, this study investigates the prevalence of NWT isolates of *S. pneumoniae* at MICs of 1 μg/mL.

Finding that the prevalence of resistance associated QRDR mutations in fluoroquinolone susceptible isolates of *S. pneumoniae* with levofloxacin MICs of 1 μg/mL was very low (0.9% of all isolates collected from 1995-1997 and 2.1% in 2003) compared to findings of previous studies looking at isolates with levofloxacin MICs of 2 μg/mL (up to 71% of isolates) supports using 1 μg/mL as a breakpoint for distinguishing wild-type fluoroquinolone susceptible isolates from NWT susceptible isolates. In addition, the lack of significant increase (P=0.34) in prevalence of mutations in this population of isolates may indicate that lowering the levofloxacin susceptible breakpoint to ≤ 1 μg/mL, where 97.9% of isolates do not possess any QRDR mutations, may prevent the selection of first step mutants. EUCAST has devised a set of wild-type epidemiological cut-off values to monitor the emergence of resistance, however for levofloxacin and *S. pneumoniae* even this value does not accurately describe the wild-type population as it is set at ≤ 2 μg/mL. CLSI currently does not have a set of breakpoints established specifically for monitoring the emergence of resistance, although microbiological breakpoints have been proposed[63, 68]. International harmonization of breakpoints would allow for improved global surveillance and standardization of reporting resistance. Levofloxacin MIC_{50} and MIC_{90}'s for *S. pneumoniae* in both North

America and in Europe is 1 μg/mL and thus lowering the clinical breakpoint to this level would not restrictively limit the use of levofloxacin for treatment of infections due to *S. pneumoniae*[59]. At the very least, the microbiological breakpoints or epidemiological cut-off values should be lowered to ≤ 1 μg/mL for more accurate monitoring of the emergence of resistance.

A major difference in the study design of this investigation compared to previous investigations on the prevalence of *parC* mutations in levofloxacin susceptible isolates[4, 63] was that the criteria for selection of isolates studied here relied on the use of a second fluoroquinolone marker ciprofloxacin. All isolates in this study were ciprofloxacin-susceptible. Previous work done in this lab has shown a strong association between ciprofloxacin resistance and the presence of single *parC* mutations[68]. Interestingly, when the same isolates were divided based on ciprofloxacin MIC (either 2 μg/mL or 1 μg/mL) a trend did appear to emerge over time (figure 3.2). Both the prevalence of isolates that had a ciprofloxacin MIC of 2 μg/mL and a levofloxacin MIC of 1 μg/mL increased from 3.9% to 6.5% (p=0.0021) and the percentage of these isolates harbouring resistance-associated mutations increased from 2.6% to 10.8% over the seven year period, although the later increase remains non-significant (p=0.12). These trends are likely a result of the increased use of all fluoroquinolones, especially ciprofloxacin which is not highly effective against *S. pneumoniae* and easily selects for single-step *parC* mutants[60]. As the use of all fluoroquinolones continues to increase it is likely that this shift to towards decreased susceptibility will continue. It is becoming increasingly important to monitor these trends and continually reassess the fluoroquinolone breakpoints.

The problem of finding an appropriate marker for assessing fluoroquinolone resistance has long been as issue. Prior to the introduction of levofloxacin, ofloxacin was used as a marker for monitoring fluoroquinolone resistance in *S. pneumoniae* in the United States. The clinical relevance of using ofloxacin as a marker was questioned because ofloxacin resistance could develop as a result of a single *parC* mutation. Ofloxacin was subsequently replaced by levofloxacin [63]. Returning to a less active compound such as ciprofloxacin that would likely be more sensitive at detecting decreased susceptibility due to first-step mutations was proposed; however, Lim *et al* found that even 29% of ciprofloxacin isolates with an MIC of 2μg/mL possess *parC* mutations [63]. The findings of this thesis suggest that using two fluoroquinolone markers, levofloxacin MIC of ≤ 1 μg/mL and ciprofloxacin MIC of ≤ 2 μg/mL, may be more effective for identifying fully fluoroquinolone-susceptible wild-type isolates than any single fluoroquinolone.

Similar results which indicate that detection of NWT isolates can best be accomplished with the use of older fluoroquinolones (norfloxacin, ciprofloxacin, pefloxacin, sparfloxacin) in conjunction with a respiratory fluoroquinolone (levofloxacin) were found when Varon *et al* recently set out to devise a nonmolecular test for the detection of NWT isolates of *S. pneumoniae* [69]. Three options for the nonmolecular test, all based on the use of fluoroquinolone disk diffusion, were recommended for use in identifying NWT isolates. The first option uses norfloxacin, ciprofloxacin, pefloxacin, sparfloxacin and levofloxacin. Based on comparative zone of inhibition sizes this test can effectively distinguish between isolates possessing topoisomerase IV mutants (ParC or ParE), DNA gyrase mutants (GyrA) and efflux mutants (as normally determined by

reserpine sensitivity). Alternatively, the proposed use of norfloxacin, pefloxacin and levofloxacin disks permits the detection of topoisomerase IV and efflux mutants, but not DNA gyrase mutants, which are exceptionally rare. Finally, the use of only norfloxacin and levofloxacin also allows for the detection of topoisomerase IV mutants and efflux mutants however does not distinguish between the two. The use of either two fluoroquinolone MICs as markers or one of the above nonmolecular disk tests could be of value in testing isolates with levofloxacin MICs of 2 μg/mL in the clinical lab to prevent possible clinical failures. Although, in Canada it is not likely of much use due to the continued low rates of fluoroquinolone resistance; this could be of great benefit to regions plagued with high rates of resistance. The use of the nonmolecular disk tests could be an excellent tool for performing future surveillance studies of the susceptible population as this would be a faster and more cost effective way of detecting the prevalence of NWT isolates than the methods used here which required sequencing every isolate.

4.1.2. Presence of an Efflux Mechanism

The presence of an efflux mechanism was also tested in the levofloxacin-susceptible isolates first by confirming the presence of the gene for the putative efflux pump *pmrA* and by the reserpine agar dilution assay. Amplification of the *pmrA* gene product occurred in all isolates with two isolates exhibiting a large deletion. A deletion in *pmrA* has previously been reported in *S. pneumoniae* NCTC 7465 (strain M4). The deletion in M4 was found to be 84 nucleotides long, which translates to a 28 amino acid deletion that was found to correspond to TMS-6 of *pmrA*[70]. The deletions found in this

study were approximately 370 bp and thus did not correspond to the deletion in M4. The deletions were not investigated further.

Despite the near universal presence of the *pmrA* gene in all the fluoroquinolone susceptible isolates of *S. pneumoniae* tested, none of these isolates exhibited a 4-fold or greater decrease in ciprofloxacin MIC when tested by agar dilution in the presence of reserpine. The significance of the presence of *pmrA* is questionable as it has been found that overexpression of *pmrA* has not been shown to be related to resistance in clinical isolates[53]. The finding that no susceptible isolates appeared to possess a reserpine-sensitive ciprofloxacin efflux mechanism via agar dilution testing is however somewhat surprising as other investigations have previously found contrasting results[71]. The use of the reserpine agar dilution assay has however yet to be validated as a method of screening for fluoroquinolone efflux and thus we cannot conclude with confidence that fluoroquinolone susceptible isolates of *S. pneumoniae* do not exhibit a fluoroquinolone efflux mechanism.

4.2. Low-Level Ciprofloxacin Resistant Isolates

4.2.1. Ciprofloxacin Efflux Assessment

Fluoroquinolone efflux as a mechanism of resistance in *S. pneumoniae* is at present poorly understood. Since no single pump has reliably been shown to efflux fluoroquinolones and contribute significantly to resistance in clinical isolates of *S. pneumoniae*, phenotypic methods of assessing efflux have been used to identify isolates as actively effluxing fluoroquinolones. Though methods vary between groups, the general concept is that increased susceptibility of an isolate to a particular fluoroquinolone in the

presence of an efflux inhibitor indicates active efflux. The second objective of this thesis was to validate the use of the reserpine agar dilution assay as an appropriate method of screening for ciprofloxacin efflux.

A select group of isolates was divided into groups based on phenotype and genotype results of the reserpine agar dilution method and QRDR sequencing. These isolates were further characterized by full gene sequencing. Those isolates that showed a 4-fold or greater decrease in ciprofloxacin MIC on the agar dilution method were assumed to be efflux positive. The full gene sequencing showed that there is a large degree of heterogeneity in all ciprofloxacin resistant isolates. No obvious association to resistance could be made between other mutations found either within the QRDRs or outside the QRDRs of ParC, ParE or GyrA; thus for the ciprofloxacin resistant isolates that did not have any known fluoroquinolone resistance-associated QRDR mutations but did have reduced susceptibility to ciprofloxacin in the presence of reserpine, efflux was initially assumed to be the major contributor to resistance.

Fluorescence accumulation assays were performed to confirm the presence of a ciprofloxacin efflux mechanism and to validate the utility of the agar dilution method as a screening tool for ciprofloxacin efflux. Wild-type isolates were used to establish a baseline level of accumulation for which to compare ciprofloxacin accumulation of the various phenotype/genotype groups. As expected, reserpine did not have any effect on ciprofloxacin accumulation in wild-type isolates. However, reserpine did not have an effect on ciprofloxacin accumulation in any of the isolates tested, including the isolates that were previously identified as efflux positive according to the agar dilution assay; thus suggesting that reserpine does not actually inhibit ciprofloxacin efflux in clinical

isolates of *S. pneumoniae.* The level of accumulation achieved by all isolates in ng ciprofloxacin per mg bacteria was higher than accumulation results reported by Piddock *et al*[46]; however, results as reported in ng ciprofloxacin per 10^9 cells were comparable to reports of ciprofloxacin accumulation by other investigators. Regardless of the units of measure reserpine had no significant effect on accumulation.

4.2.1.1. Identification of Putative Efflux Pump PatA/B

The lack of effect of reserpine on ciprofloxacin accumulation has been previously reported by Piddock *et al* and also agrees with recent results published by Marrer *et al*[70]. Using DNA microarray analysis of strain M4 and a multidrug resistant derivative of M4 named M22, Marrer *et al* recently identified two genes, *patA* and *patB*, as potential fluoroquinolone efflux pumps with sequence homology to ABC transporter proteins[64]. Expression levels of *patA* and *patB* were induced by ciprofloxacin in both strains of *S. pneumoniae,* however M4 expression of these genes only reached expression levels of M22 after prolonged ciprofloxacin exposure. Ciprofloxacin accumulation in M22 was approximately half the accumulation in parent strain M4, and knock out mutants of *patB* restored accumulation to M4 levels. Similar to the results presented in this thesis, it was found that although susceptibility was increased in the presence of reserpine, accumulation was not affected by reserpine. Reserpine was found to have a marked effect on gene expression, with 60 genes being strongly repressed by reserpine. More importantly, of those 60 genes 34 were induced by ciprofloxacin, suggesting that reserpine may not directly inhibit efflux, but instead acts by antagonizing gene expression changes that occur during exposure to ciprofloxacin[64].

4.2.1.2 The Reserpine Effect

Further investigations by the same group found that several genes involved in the SOS repair system were overexpressed in the presence of ciprofloxacin in M4[64]. Of note the *hexA* and *hexB* genes that form part of the mismatch repair system in *S. pneumoniae* were upregulated in M4 in the presence of ciprofloxacin however were constitutively repressed in M22[64]. This might indicate the possibility of M22 being a hypermutator strain due to the constant repression of genes involved in DNA repair.

To assess whether increased susceptibility to ciprofloxacin in the presence of reserpine, regardless of reserpine's inability to directly inhibit efflux, was nonetheless an indicator of an efflux mechanism, average levels of accumulation among the various phenotypic groups were assessed. If reserpine did in fact identify isolates with an active efflux mechanism we would expect the isolates with positive reserpine agar dilution results to accumulate less ciprofloxacin than those isolates with negative reserpine agar dilution results. The results shown in figure 3.7 show that no difference was seen in the average 5 minute accumulation levels amongst the various phenotypes; thus indicating that reserpine is not a good predictor of an active efflux mechanism.

Additionally, to determine whether reserpine was identifying isolates with a hypermutator phenotype, the average number of amino acid substitutions per subunit of each phenotype was assessed, as well as, the average number of amino acid substitutions in all 3 subunits. No significant difference was seen in any individual subunit or in the overall average number of amino acid substitutions. The reserpine-sensitive clinical

isolates do not appear to have an increased number of mutations compared to the isolates that are not affected by reserpine.

4.3. Conclusions

1. The prevalence of single-step *parC* mutations in isolates with a levofloxacin MIC of 1 μg/mL is low (2.1% of isolates from 2003) compared to the prevalence in isolates with levofloxacin MICs of 2 μg/mL previously reported as high as 71%; thus confirming the hypothesis that the CLSI levofloxacin susceptibility breakpoint of ≤ 2 μg/mL is not able to effectively distinguish between wild-type susceptible isolates and NWT susceptible isolates. Whereas, at a levofloxacin MIC of 1 μg/mL the probability of encountering a NWT isolate is very low. As such, the levofloxacin susceptibility breakpoint should be lowered to 1 μg/mL for *S. pneumoniae* in order to reduce the probability treatment failure due to the presence of isolates possessing single-step *parC* mutations which rapidly acquire second-step *gyrA* mutations and become fully resistant to all fluoroquinolones.

2. The use of two fluoroquinolone markers, including one older fluoroquinolone which can acquire resistance as a result of a single mutation and one respiratory fluoroquinolone, serves as a better indicator than the use of a single fluoroquinolone marker for detecting NWT isolates.

3. Despite the antagonizing effect to ciprofloxacin in some isolates of *S. pneumoniae,* reserpine does not act as a direct inhibitor of ciprofloxacin accumulation nor

does it identify hypermutators. This addresses the second objective of this thesis by invalidating the use of the reserpine agar dilution assay as an appropriate tool for screening for efflux clinical isolates of *S. pneumoniae.* The effect reserpine has on fluoroquinolone resistance remains unknown and the results of this assay, as well as similar assays relying on reserpine for identifying efflux, should be regarded as questionable until the role of reserpine is fully elucidated.

The conclusions of this thesis have raised more questions about the role reserpine plays in antagonizing fluoroquinolone resistance and about the role of efflux. The experiments performed here have failed to confidently identify any clinical isolates as efflux positive; begging the question 'does fluoroquinolone efflux play a role in contributing to resistance in clinical isolates of *S. pneumoniae*?'

4.4. Future Directions

The majority of research in the field of fluoroquinolone efflux in *S. pneumoniae* has been performed on lab generated efflux mutants that have been shown to accumulate significantly less ciprofloxacin or norfloxacin than their parent strains. As recent findings from two separate groups using two different strains have simultaneously identified the *patA* and *patB* genes as encoding heterodimeric subunits of a putative fluoroquinolone efflux pump belonging to the ABC transporter family the potential for research in this field is increased and can become more focused. Nevertheless, the results obtained using lab mutants must be applied to clinical isolates in order to assess any real relevance. Potential avenues to explore are as follows:

1. To further investigate the use of the agar dilution assay, gene expression studies such as the DNA microarray assays performed by Marrer *et al*[64] could be performed on various phenotypic groups of clinical isolates in order to identify any major differences in gene expression. The results of these studies should be compared back to the lab strains M4 and its efflux mutant M22 to determine the relevance of using these lab strains for further investigations.

2. The role of PatA/B can be assessed by creating both knockout mutants and mutants that overexpress these genes from clinical isolates. Again, comparing these results among various phenotypes may play an important role in advancing the understanding of the effect of reserpine.

3. Depending on the results of further investigations of PatA/B, if this pump is in fact a contributor to fluoroquinolone resistance screening for efflux inhibitors should be performed. The identification of an effective efflux inhibitor will assist in the development of novel techniques for identifying efflux in clinical isolates.

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