Investigation of Fluoroquinolone Resistance-Associated Mutations in Canadian Clinical Isolates and Laboratory Mutants of *Streptococcus pneumoniae*

By

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A Thesis Submitted to the Faculty of Graduate Studies

in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

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Abstract

The purpose of this thesis was to evaluate how *S. pneumoniae* isolates are genetically altered as fluoroquinolone resistance increases in Canada. In order to address this question, it is essential that the fluoroquinolones be fully characterized for activity, resistance development, and mechanisms to prevent the selection of resistant isolates.

This study was comprised of 3 objectives. Firstly, to molecularly characterize ciprofloxacin-resistant isolates collected over 7 years for resistance-associated substitutions, efflux, serotype, and molecular subtyping using pulsed-field gel electrophoresis. Secondly, to ascertain if the prevalence of fluoroquinolone resistance-associated substitutions has increased in fluoroquinolone-susceptible isolates. Lastly, the mutant prevention concentration (MPC) was evaluated for its potential to restrict the selection of fluoroquinolone-resistant isolates during antibiotic therapy.

S. pneumoniae isolates were collected as part of the Canadian Respiratory Organism Susceptibility Study between 1997/98 and 2004. Fluoroquinolone resistance was found to primarily result from the spontaneous development of resistance substitutions, not clonal expansion. An increase was observed in the number of isolates with substitutions in both target enzymes in conjunction with a decrease in the number of efflux-positive isolates.

The percent of fluoroquinolone-susceptible isolates with fluoroquinolone resistance-associated substitutions increased in Canada between 1997 and 2003. Using this data, microbiological breakpoints were determined to separate wild-type *S. pneumoniae* isolates from those considered susceptible by standard susceptibility criteria but possessing resistance-associated substitutions. Microbiological breakpoints permit a

means to conduct surveillance of genetic alterations resulting in fluoroquinolone resistance.

The MPC has been proposed as a dosing strategy that may limit the selection of resistance during antimicrobial therapy. It was demonstrated throughout this study that the MPCs vary widely based on the original genetic makeup of the *S. pneumoniae* isolate.

Fluoroquinolone resistance has increased in Canada throughout the course of this study and the ciprofloxacin-resistant isolates were found to be genetically evolving. The resistance increase does not appear to be attributable to a pooling of resistance substitutions in fluoroquinolone-susceptible isolates. MPC dosing may be effective at limiting the selection of fluoroquinolone resistance from a wild-type population, but will be unable to remedy the difficulties recently highlighted with *S. pneumoniae* isolates carrying first-step substitutions.

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List of Abbreviations

ATCC	American Type Culture Collection
bp	Base pair
CFU	Colony forming unit
Cipro	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
CROSS	Canadian Respiratory Organism Susceptibility Study
CSP	Competence-stimulating peptide
EDTA	Ethylenediamine-tetra-acetic acid
EUCAST	European Committee on Antimicrobial Susceptibility Testing
Gati	Gatifloxacin
Gemi	Gemifloxacin
Kb	Kilobase pairs
Levo	Levofloxacin
MIC	Minimum inhibitory concentration
mL	Millilitre
mM	Millimolar
Moxi	Moxifloxacin
MPC	Mutant prevention concentration
MP_{MIC}	Mutant prevention concentration as a fold increase of the MIC
ng	Nanogram
OD	Optical density
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PK	Pharmacokinetics
PD	Pharmacodynamics
QRDR	Quinolone resistance-determining region
QTc	Rate-corrected QT interval
SBA	Trypticase soy agar with 5% sheep blood
THYB	Todd-Hewitt Broth with 5% yeast extract
µg	Microgram(s)
µL	Microlitre(s)
µm	Micrometer(s)
µM	Micromolar
V	Volts
vol/vol	Volume/volume

A. INTRODUCTION

1. *Streptococcus pneumoniae*

a. Characteristics of *S. pneumoniae*

i. Colony Morphology and the Polysaccharide Capsule

Streptococci are gram-positive, catalase-negative, facultatively anaerobic bacteria that are spherical or ovoid in shape, replicate in pairs or short chains, and are usually less than 2 μm in diameter (54, 88). *S. pneumoniae* belong to the viridans group streptococci and are differentiated from the rest of the viridans group by their bile solubility and optochin sensitivity (108). Ideally, *S. pneumoniae* are grown on complex media that is enriched with blood and incubated at 35 to 37°C in an atmosphere containing 5% CO_2 (54, 108). *S. pneumoniae* display α-hemolysis on blood agar, which is visualized as a green discoloration of the media due to the pneumolysin, formerly referred to as α-hemolysin, breaking down the hemoglobin in the media (54, 108). *S. pneumoniae* colonies vary in color from gray to whitish and may appear mucoid due to the production of capsular polysaccharide (88).

Almost every *S. pneumoniae* isolate has a polysaccharide capsule. The polysaccharide capsule is the primary virulence factor of *S. pneumoniae* (88). The polysaccharide capsule resists phagocytosis and activates complement (88). There have been ninety serotypes of *S. pneumoniae* identified on the basis of the antigenic differences of their capsules (88). It has been demonstrated that *S. pneumoniae* can switch capsular types. Capsular switching is believed to occur by natural transformation of large DNA fragments involving the capsular biosynthesis locus (126). Capsular switching from 6B to 23F, 15B to 15C, 9V to 14, and 23F to 3, 6B, 9N, 9V, 11, 14, 19A,

and 19F have been previously identified (64, 106, 126). As vaccine development has focused on targeting the most commonly observed serotypes, an organism's ability to switch serotypes may decrease the vaccine protective effect.

ii. Natural Transformation of *S. pneumoniae*

The active uptake of free DNA and the incorporation of the genetic information is a mechanism of horizontal gene transfer in bacteria that is referred to as natural transformation. Transformation was first described by Griffith in 1928 with strains of *S. pneumoniae* (52). Since that time, a considerable amount of work has been conducted to determine the mechanisms by which transformation occurs.

In *S. pneumoniae*, DNA uptake during transformation proceeds in two key stages: binding of double-stranded DNA to the cell and entry of single-stranded linear DNA into the cell (13, 74, 133). EndA is the nuclease in *S. pneumoniae* that degrades one DNA strand while the complement strand is internalized in a 3' to 5' direction into the cell (13, 133). The internalized DNA is bound and protected from degradation by single-stranded binding proteins (74, 133). Homologous DNA sequences are recombined into the chromosome (74, 133).

Transformation requires cells to be in a state of genetic competence. Competence is a transitory state that occurs in all pneumococcal cells in a culture during the exponential phase of growth (29). Genetic competence occurs at a cell density of approximately 10^7 cells/mL and involves a change in protein synthesis (29, 56). During this state, the expression of approximately 150 genes transiently increases (81). Competence is affected by the pneumococcal strain, composition of the media, pH, temperature, and concentrations of calcium, magnesium and phosphate (29, 74).

Competence regulation requires a quorum-sensing system, *comABCDE*. The extracellular heptadecapeptide referred to as the competence-stimulating peptide (CSP) induces the expression of genes involved in transformation (56). Recent studies have demonstrated that both cell-produced and synthetic CSP can induce competence *in vitro* when the concentration of CSP is between 1 and 10 ng/mL (56, 105). CSP is encoded by the 3' end of *comC* (29). Two alleles of *comC*, *comC1* and *comC2*, encode CSP-1 and CSP-2, which differ at eight residues (105). The vast majority of *S. pneumoniae* strains only respond to the CSP type encoded by their *comC* allele (105). CSP is cleaved and transported extracellularly by an ATP-binding cassette transporter, ComAB (30, 73, 74, 133). An extracellular accumulation of CSP signals the sensor ComD, a membrane-located histidine protein kinase, to phosphorylate the response regulator ComE (30, 73, 74, 133). ComE is activated by phosphorylation and it then regulates 2 operons of the quorum-sensing system and approximately 8 other operons (81). ComE binds to the *comAB* and *comCDE* operons and activates their transcription via a positive feedback loop (30, 74). ComE also activates the transcription of *comX* and *comW* (30, 73, 74, 81). ComX provides a link between the quorum-sensing system and the genes necessary for genetic transformation (73, 74, 81). ComW has recently been shown to be required for optimal production of ComX, although it's exact role in competence regulation has yet to be elucidated (81). ComX and ComW also appear to play a role in the down-regulation of competence although these processes remain largely unknown (81).

b. Infections and Carriage of *S. pneumoniae*

The normal ecological niche of *S. pneumoniae* is the nasopharynx of healthy individuals. The nasopharynx is a stable environment for *S. pneumoniae* permitting both

colonization and the opportunity to spread to other hosts via aerosols and mucus. Generally, 5 to 10% of healthy adults and 20 to 40% of healthy children are colonized (88). In order to cause infection, strains spread from the nasopharynx to normally sterile sites. The overall worldwide rate of invasive pneumococcal disease is approximately 15 per 100,000 persons per year (88). The rates of invasive disease are high in newborns and children less than 2 years of age, significantly less in older children and young adults, and increase again in adults over 65 years of age (88). Numerous risk factors have been identified that predispose individuals to infection, including defective antibody formation, defective complement, insufficient or poorly functioning polymorphonuclear leukocytes, excessive exposure as in daycares, prior respiratory infections or underlying inflammatory conditions (88).

S. pneumoniae is a primary pathogen of acute exacerbations of chronic bronchitis, meningitis, otitis media, pneumonia, and sinusitis (53, 113, 123, 125). Pneumonia is one of the leading causes of death worldwide. Approximately 4.5 million adults in the United States suffer from community-acquired pneumonia annually (125). Between 8 and 51% of individuals with community-acquired pneumonia will require hospitalization (131) and of those, up to 35% may die (125). Pneumonia most commonly affects individuals at the extremes of age (131). *S. pneumoniae* is responsible for the majority of cases of community-acquired pneumonia. *S. pneumoniae* infections cause significant morbidity and mortality worldwide.

Effective treatment of *S. pneumoniae* infections are crucial as lower respiratory tract infections, generally caused by *S. pneumoniae*, are one of the most common causes of death from infectious diseases worldwide.

c. Empiric Therapy of *S. pneumoniae*

Respiratory tract infections are generally treated empirically. The antimicrobial chosen for therapy must cover the likely pathogens and reflect the increase in resistance to common agents worldwide (44). Infections likely caused by *S. pneumoniae* are frequently treated with penicillins and macrolides as these are generally effective and safe antimicrobials.

Prior to the mid-1970s, *S. pneumoniae* isolates were susceptible to all pertinent antimicrobials. A *S. pneumoniae* isolate with reduced penicillin susceptibility was first reported in the United States in 1965 (71). The prevalence of penicillin non-susceptible *S. pneumoniae* isolates remained low in the United States until the 1990s (9). Subsequently, penicillin non-susceptibility has increased substantially to 34.2% (38). Penicillin resistance has also increased in Canada, although it remains lower than in the United States. Penicillin resistance (minimum inhibitory concentration (MIC) $\geq$ 2 μg/mL) and non-susceptible (MIC $\geq$ 0.12 μg/mL) rates in Canadian *S. pneumoniae* isolates increased from 6.6% and 21.5% in 1997/98 to 9.6% and 25.3% in 2004 (updated from (136)). Multi-drug resistance is frequently reported among the penicillin-resistant pneumococci. Pneumococci are considered multi-drug resistant if they are resistant to penicillin and 2 other antimicrobials from different drug classes (107). In highly penicillin-resistant isolates, significant proportions are also resistant to antimicrobials such as chloramphenicol, tetracycline, trimethoprim-sulfamethoxazole, macrolides, and often the cephalosporins (22, 38, 68, 82). The increase in multi-drug resistant isolates severely limits treatment options.

In conjunction with the increased rates of penicillin-resistant *S. pneumoniae* worldwide, macrolide resistance has increased. Macrolide resistance (MIC $\geq$ 1 μg/mL for erythromycin or clarithromycin) increased substantially during the 1990s such that over 30% of clinical isolates of *S. pneumoniae* are resistant in some regions (60, 68). In Canada, the prevalence of macrolide-resistant *S. pneumoniae* increased from 5.8% in 1997/98 to 13.4% in 2004, using clarithromycin as the marker (updated from (136)).

The increase in penicillin and macrolide resistance in *S. pneumoniae* has lead to the recommendation of fluoroquinolones for use as either empiric first-line therapy for patients with community-acquired pneumonia or for use specifically against penicillin and macrolide-resistant *S. pneumoniae* (24, 44). Accordingly, fluoroquinolones are increasingly used in the treatment of respiratory tract infections.

Fluoroquinolone resistance is generally low; however, high rates of fluoroquinolone-resistant *S. pneumoniae* have been observed in countries like Spain (5.3%) (49), Hong Kong (12.1%) (62), and Ireland (15.2%) (51). This highlights the importance of both appropriate use of the fluoroquinolones and a continuation of worldwide surveillance.

Fluoroquinolone resistance is generally monitored based on ciprofloxacin MICs. Although the Clinical and Laboratory Standards Institute (CLSI) (formerly the National Committee for Clinical Laboratory Standards) does not provide breakpoints for *S. pneumoniae* and ciprofloxacin, MIC $\geq$ 4 μg/mL is recommended as a phenotypic measure of fluoroquinolone activity (27). As the majority of *S. pneumoniae* have ciprofloxacin MICs $\leq$ 2 μg/mL, an MIC $\geq$ 4 μg/mL is useful for the detection of upward shifts in MICs (38). Additionally, quinolone resistance-associated mutations begin to accumulate at a

ciprofloxacin MIC of 4 μg/mL (38). Thus, *S. pneumoniae* isolates with ciprofloxacin MICs ≥ 4 μg/mL are considered ciprofloxacin-resistant.

Surveillance of fluoroquinolone resistance in *S. pneumoniae* is then possible by routinely collecting specimens and determining their ciprofloxacin susceptibility. The surveillance observations can provide a basis for treatment decisions.

In order to maintain the efficacy of the fluoroquinolones in light of their increased usage, targeted prescribing focusing on appropriate use must be strongly advocated. Increased fluoroquinolone use has been associated with increased non-susceptibility, which can lead to treatment failures (27, 35, 48, 129). The fluoroquinolones should be reserved for use with patients who are allergic to β-lactams, are infected with a multi-drug resistant strain, failed on initial therapy, have had steroids or had previous antimicrobial therapy with another antimicrobial class in the last 3 months (10, 60). In combination with appropriate fluoroquinolone usage, the vaccination of children with the pneumococcal conjugate vaccine should be recommended in order to limit antimicrobial use and resistance development (79).

2. Fluoroquinolones

a. History and Development

The quinolones are often categorized into 4 generations on the basis of their spectrum of activity (128). The quinolone class of antimicrobials originated with the discovery of nalidixic acid in 1962 (5, 21, 110, 128, 135). Nalidixic acid is known as the prototype quinolone because it was the first to display the 4-quinolone structure (110). It was one of the first antibacterials known to function by inhibition of DNA

replication (70). Nalidixic acid displayed good activity against Gram-negative aerobes; however, it had poor activity against Gram-positive organisms, demonstrated only modest serum and tissue concentrations, had fairly high MICs, and had associated toxicity issues (110, 128, 135). Further development of quinolones yielded minimally improved antibimicrobials such as cinoxacin and pipermidic acids (21). The early quinolones were mainly used for the treatment of urinary tract infections (5).

The catalyst for increased quinolone research was the improved pharmacokinetic properties associated with the discovery of norfloxacin and ciprofloxacin in the 1980's (135). Norfloxacin and ciprofloxacin displayed a broader spectrum of activity against both Gram-negative and Gram-positive organisms and were much more potent than their progenitor antimicrobials (17, 110, 135). These agents contain a piperazine substitution at position 7 and a fluorine atom at position 6 (17, 110). The addition of the fluorine atom lead to the naming of these antibacterial agents as fluoroquinolones (17, 110). Ciprofloxacin, norfloxacin, and ofloxacin comprise the second generation of quinolones (128).

Improvements have continued to be made to the fluoroquinolone class via the addition of various molecular substituents to the basic quinolone structure yielding the third-generation quinolones. The newer fluoroquinolones, gatifloxacin, gemifloxacin, levofloxacin, and moxifloxacin, are referred to as respiratory fluoroquinolones because they have enhanced activity against Gram-positive organisms like *S. pneumoniae* (17, 128). Additionally, gatifloxacin and moxifloxacin display increased anti-anaerobe activity (17, 128). The improved activities of the third-generation molecules are due to

the presence of an alkyl-substituted piperazine or pyrrolidine at position 7 and a methoxy at position 8 (gatifloxacin and moxifloxacin) (128).

The fourth generation of quinolones is referred to as des-fluoroquinolones because the fluorine atom at position 6 has been removed (128). The representative quinolone of the fourth generation is garenoxacin although many others are currently in development.

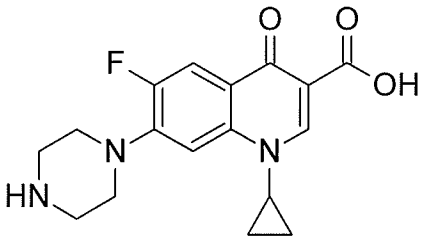
The quinolones have been the subject of considerable study as they potentially possess many characteristics of an ideal antimicrobial: high potency, broad spectrum of activity, good bioavailability, availability of oral and intravenous formulations, high serum concentrations, extensive tissue distribution, and the possibility of a low frequency of side-effects (5).

b. Structure

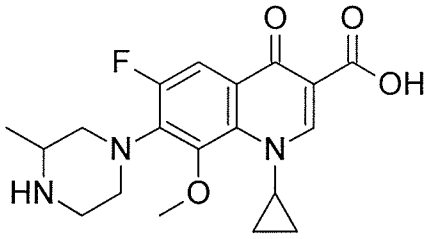
The structures of the currently available fluoroquinolones, ciprofloxacin, gatifloxacin, gemifloxacin, levofloxacin, and moxifloxacin, and the representative des-fluoroquinolone, garenoxacin, are presented in figure 1.1. Fluoroquinolones are based on a 4-quinolone nucleus comprised of a nitrogen-containing 8-membered heterocyclic aromatic ring (21, 110). The structure consists of a dual-ring with nitrogen at position 1, a carboxyl group attached to carbon 3, a carbonyl group at position 4, and a fluorine at position 6 (21). The carboxyl and carbonyl groups are essential for activity as they mediate both the transportation of the fluoroquinolone into the bacterial cell and the binding of the fluoroquinolone to the DNA-DNA gyrase complex (135). The fluorine at position 6 influences both the drug's activity and its binding to the DNA-DNA gyrase complex (135). This single structural alteration improved the inhibition of gyrase by 10-

Figure 1.1 **Chemical structures of ciprofloxacin, gatifloxacin, garenoxacin, gemifloxacin, levofloxacin, and moxifloxacin.**

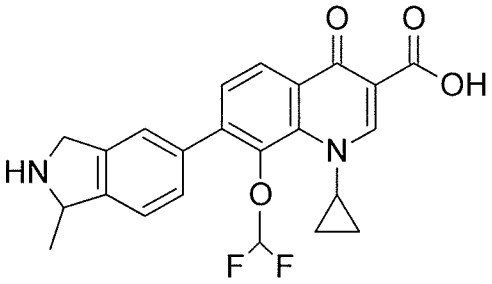
Fig. 1.1



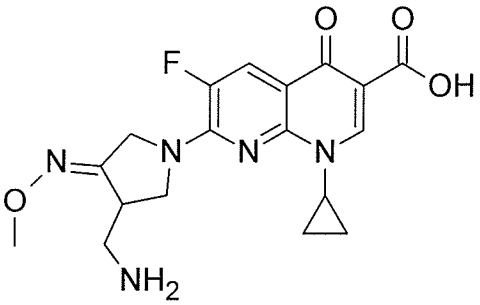
Ciprofloxacin



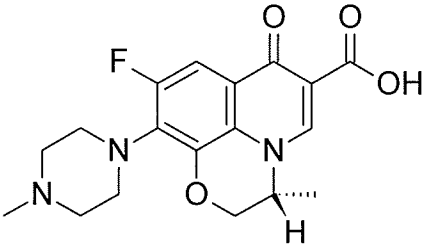
Gatifloxacin



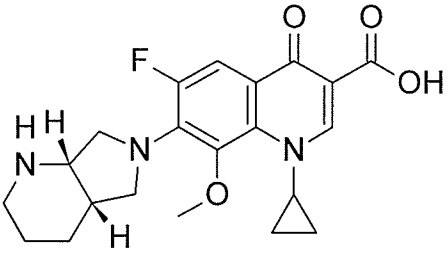
Garenoxacin



Gemifloxacin



Levofloxacin



Moxifloxacin

fold and provided up to a 100-fold improvement in MIC (5). Structural alterations at each site on the quinolone structure have been analyzed for activity and toxicity relationships. The relationships between various common substituents at each position on the quinolone structure are displayed in figure 1.2.

i. Position 1

Substituents of position 1 are part of the enzyme-DNA binding complex (100). A cyclopropyl ring at position N1, as seen in ciprofloxacin, gatifloxacin, garenoxacin, gemifloxacin, and moxifloxacin, has been shown to enhance the overall activity of the antimicrobial (5, 77). A 2,4-difluorophenyl group was added at position 1 in trovafloxacin and temafloxacin and was shown to enhance activity, in particular against anaerobes (5). Subsequently, the 1-(2-4)-difluorophenyl substituent has been associated with severe immunologically-mediated adverse reactions (5).

ii. Position 2

Position 2 is usually hydrogen as it is close to the binding site of DNA gyrase and topoisomerase IV. Any increase in the bulk of this substituents results in a decrease in activity (100).

iii. Positions 3 and 4

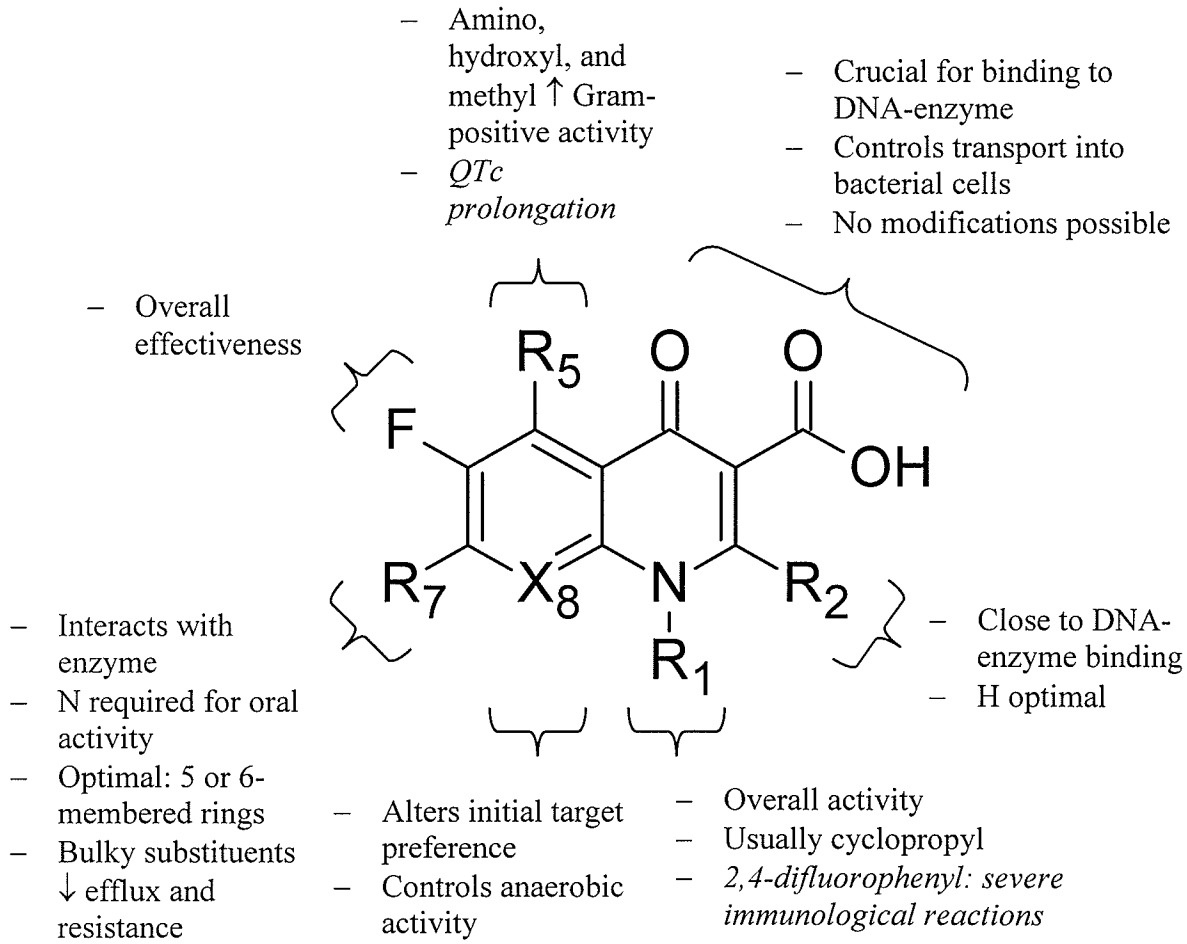
Both position 3 and 4 are crucial for binding to cleaved DNA therefore they cannot be altered from the 3-carboxylate and 4-carbonyl groups of the basic quinolone structure (100).

iv. Position 5

Substituents of position 5 alter the overall stearic configuration of the quinolone, which affects activity (100). Amino, hydroxyl and methyl groups at position 5 augment

Figure 1.2 **The structure-activity and toxicity relationship of substituents at each position on the quinolone molecule. The activity is indicated in regular script and the toxicity is indicated in italics. ↑, increase; ↓, decrease. Adapted from (39).**

Fig. 1.2


– Amino, hydroxyl, and methyl ↑ Gram-positive activity
– *QTc prolongation*
– Crucial for binding to DNA-enzyme
– Controls transport into bacterial cells
– No modifications possible
– Overall effectiveness
– Interacts with enzyme
– N required for oral activity
– Optimal: 5 or 6-membered rings
– Bulky substituents ↓ efflux and resistance development
– *Small piperazine rings: central nervous system reactions*
– Alters initial target preference
– Controls anaerobic activity
– Halogen, methyl or methoxy ↑ Gram-positive activity
– *Phototoxicity*
– Overall activity
– Usually cyclopropyl
– *2,4-difluorophenyl: severe immunological reactions*
– Close to DNA-enzyme binding
– H optimal

the overall effectiveness of the antimicrobial against Gram-positive organisms (77, 100). Substitutions at position 5 may play a role in the prolongation of the rate-corrected QT interval of the heart rate, QTc (5).

v. Position 6

The addition of the fluorine at position 6 resulted in a significant increase in activity and gave rise to the naming of this antimicrobial group (100). A halogen at position 6 improves the overall activity of the antimicrobial (77). Quinolones have also been developed with a hydrogen or amino group at position 6 (100). The activity of these compounds is determined by the substituents of positions 1, 7, and 8 (100).

vi. Position 7

This position directly interacts with DNA gyrase and topoisomerase IV (100). Substituents of position 7 are correlated with activity and bioavailability (5, 135). The optimal groups at this position are 5 or 6-membered nitrogen heterocyclic rings (100). A pyrrolidine ring added at position 7, as in gemifloxacin, enhances the effect on Gram-positive organisms (5, 77, 100, 135). Unfortunately, the pyrrolidine rings have been associated with low water solubility and oral bioavailability (5). Alternatively, the addition of a piperazine, as in ciprofloxacin, gatifloxacin, levofloxacin and norfloxacin, improves the activity against Gram-negative organisms (5). Small piperazine substituents have been associated with a higher incidence of central nervous system adverse reactions (7). The addition of methyl groups to the pyrrolidine and piperazine rings has been shown to improve oral absorption and *in vitro* activity (5). Bulky substituents of position 7 have been associated with decreased efflux, decreased

resistance development, and increased anti-anaerobic activity (100). Of the currently available fluoroquinolones, moxifloxacin has the bulkiest substituent (100).

vii. Position 8

Similarly to position 5, the substituents of position 8 affect the overall stearic configuration of the fluoroquinolone (100). The substituents of this position have been shown to affect the initial target preference, DNA gyrase or topoisomerase IV, of the fluoroquinolone (100). Substituents of position 8 have been associated with altered oral pharmacokinetics, improved spectrum of activity, increased tissue penetration, extended half-life of the antimicrobial, and reduced mutant selection (5). Halogen, methyl or methoxy groups increase the activity against Gram-positive cocci (100). A second halogen located at position 8 enhances the activity against anaerobic organisms, but may cause phototoxicity (77, 100, 135). Methoxy groups at this position, as seen in gatifloxacin and moxifloxacin, have been shown to be the least likely to cause phototoxicity (5).

Throughout the development of the fluoroquinolones, a few promising agents have caused severe adverse reactions resulting in either their complete removal or severe restriction of their clinical use. These fluoroquinolones include clinafloxacin, grepafloxacin, sparfloxacin, temafloxacin, and trovafloxacin (7). Although some potentially severe toxicities are highlighted in this section, the adverse effects of the fluoroquinolone class are usually minor, including gastrointestinal disorders, central nervous system and skin disturbances (7). Of the currently available fluoroquinolones, ciprofloxacin, levofloxacin, and moxifloxacin have low levels of associated adverse

reactions and are considered very safe. There are increasing numbers of reports and concerns about glucose disturbances in patients treated with gatifloxacin (15).

c. Mechanism of Action

i. Function of DNA gyrase and topoisomerase IV

Fluoroquinolones enter *S. pneumoniae* by passive diffusion through the cytoplasmic membrane (134). Fluoroquinolones target two essential enzymes, DNA gyrase and topoisomerase IV, which are essential for replication. Topoisomerases maintain the integrity of the genetic material during replication by altering the topological state of DNA. DNA gyrase and topoisomerase IV are type II topoisomerases.

Type II topoisomerases modulate over-winding and under-winding of DNA and separate interlocked daughter chromosomes by creating a double-stranded break in the DNA, passing a second duplex through the break and re-ligating the broken strands in an ATP-dependent reaction (14, 91).

DNA gyrase and topoisomerase IV are A_2B_2 heterotetramers encoded by *gyrA* and *gyrB* and *parC* and *parE*, respectively. Considerable homology exists between the A subunits, *gyrA* and *parC*, and the B subunits, *gyrB* and *parE*. The A subunits are responsible for DNA binding, cleavage, and religation (75). The B subunits are responsible for ATP binding and hydrolysis (75).

DNA gyrase is essential for the maintenance of DNA topology during transcription, initiation, and elongation (75). DNA gyrase can relax positive supercoils as well as form negative supercoils (31). Topoisomerase IV is also able to relax positive supercoils and is responsible for the decatenation of sister chromatids following a round

of replication (31). DNA gyrase may be able to perform a portion of the functions of topoisomerase IV, but the reverse is not true (75).

Although DNA gyrase and topoisomerase IV are very similar, the differences in their functions are related to different interactions between the enzymes and DNA. The ability of DNA gyrase to generate negative supercoils ahead of the replication fork is a result of its ability to wrap DNA around itself in a right-handed coil (75). DNA gyrase thus preferentially acts on intramolecular reactions and prevents the stalling of the replication fork by topological stress (43, 75). Conversely, topoisomerase IV does not wrap DNA around itself and thus prefers intermolecular reactions (43, 75). Topoisomerase IV binds preferentially to left-handed DNA crossovers that are common in positive supercoils and the hooked geometry of catenated DNA (31). In this way, DNA gyrase imposes the necessary crossover geometry for its function whereas topoisomerase IV recognizes, but does not create, its preferred geometry (31).

The specific mechanisms of the binding, cleaving, and re-ligating necessary for the function of DNA gyrase and topoisomerase IV have been determined. Topoisomerases cleave the DNA by forming a transient, covalent phosphotyrosine enzyme – DNA bond (14). Two tyrosines, Tyr122 in GyrA and Tyr120 in ParC, attack opposite strands of the DNA duplex and become covalently attached to the DNA via the phosphotyrosine links (14, 75). The cleavage generates staggered cuts with free 3' hydroxyl ends and 4 base pair (bp) 5' overhangs (75). The mechanism of action of the type II topoisomerases is described by a two-gate model. The enzyme binds a DNA duplex referred to as the G (DNA gate) segment (14, 31). A second duplex, the transport duplex (T), enters through a gate on one side of the enzyme (14, 31, 75). The G segment

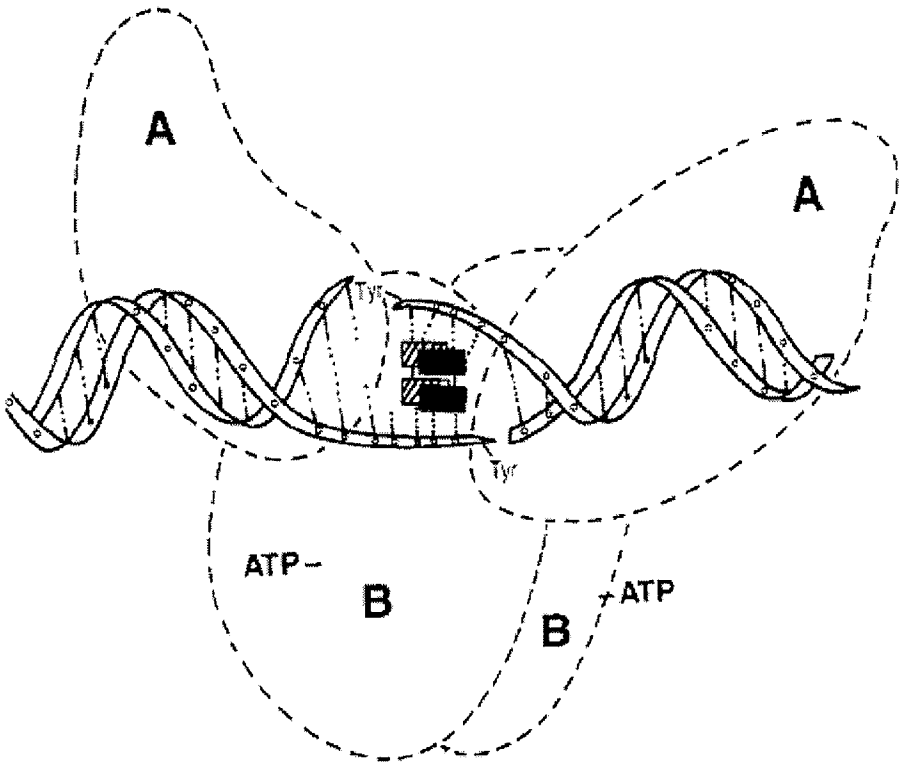
is cleaved and remains covalently attached to the enzyme by phosphotyrosine bonds (75). The T segment passes through the break in the G duplex (14, 31, 75). The T segment then exits the enzyme through a second gate on the other side of the enzyme (14, 31, 75).

ii. Inhibition of DNA gyrase and topoisomerase IV by the fluoroquinolones

Fluoroquinolones block DNA synthesis. The activity of fluoroquinolones is a result of the formation of stabilized complexes of DNA, DNA gyrase or topoisomerase IV, and the quinolone molecules, which is referred to as the cleavable complex (43, 46, 58, 70). The seminal aspect of the cleavable complex is it contains broken DNA. A quinolone binding model has been proposed by Shen *et al.* and is portrayed in figure 1.3 (114). The quinolones are believed to bind between the A and B subunits of the enzyme and stack within the DNA molecules (110). Although Shen's model proposed the association of 4 quinolone molecules to a bubble in double-stranded DNA, recent stoichiometry results suggest that 2 fluoroquinolone molecules most likely bind per complex (32). In the model proposed by Heddle and Maxwell, two molecules of ciprofloxacin bind between DNA bases at the active site of DNA gyrase where the DNA is distorted (59). They suggest that on either side of the duplex, one ciprofloxacin molecule either intercalates between bases or binds to a guanine and "flips out" the cytosine (59). Recent crystal work indicates that the quinolones disrupt the terminal base pair and stack on the penultimate C:G base pair (115). Regardless of recent suggestions, the Shen model remains the most widely held model. The fluoroquinolones have a high affinity for self-association and may bind by tail-to-tail hydrophobic interactions between their N1 groups (28). This self-association permits the formation of hydrogen bonds

Figure 1.3 **Shen's proposed quinolone-DNA binding model, as presented diagrammatically by Shen (114). The A and B subunits of the enzyme are marked as "A" and "B". Four quinolone molecules are indicated by the rectangles bound to the cleaved DNA.**

Fig. 1.3



between the 3-carboxyl and 4-carbonyl groups of the quinolones and both strands of the DNA duplex via Mg^{2+} ions (110). The bound quinolones prevent the re-ligation of the broken DNA strands (57).

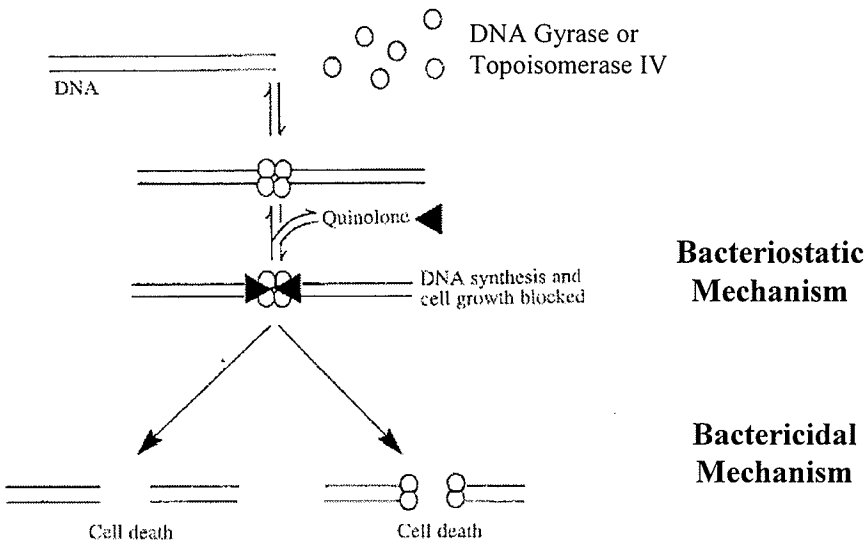
Following the formation of cleavable complexes, the quinolones may act by either a bacteriostatic or bactericidal mechanism. Typically, lower concentrations of quinolones act by a bacteriostatic mechanism whereas high concentrations of quinolones are bactericidal (111). A diagrammatic representation of the bacteriostatic and bactericidal mechanisms is presented in figure 1.4. Two potential bactericidal mechanisms are portrayed. One shows the release of broken DNA from the cleavable complex and the other shows the quinolone molecules forcing apart the enzyme-DNA complex (26, 43).

The bacteriostatic action of quinolones results from the reversible inhibition of DNA synthesis and cell growth due to the formation of the cleavable complexes (43, 57). The formation of the cleavable complex blocks DNA synthesis and cell growth by preventing the normal enzymatic activity of DNA gyrase or topoisomerase IV. The enzymes are unable to release the superhelical tension resulting from replication (26).

The stabilization of the enzyme-DNA complex is insufficient to explain the bactericidal activity of the fluoroquinolones. The bactericidal action of quinolones occurs as topoisomerases are converted to cellular toxins releasing double-stranded breaks in the DNA (91). Two models have been proposed to describe the release of double-stranded DNA breaks: fork collision and dispersed complexes model. In the fork collision model, the cleavable complexes are converted to a non-reversible form subsequent to a collision with the replication fork (75, 111). In the dispersed model, lethal breaks are released from complexes dispersed throughout the chromosome (111).

Figure 1.4 **Diagrammatic representation of the bacteriostatic and bactericidal mechanisms of quinolone-mediated cell death. Adapted from (26). DNA is represented by two parallel lines. The enzyme is represented by four circles. The quinolone molecules are represented by two black triangles.**

Fig. 1.4


DNA Gyrase or
Topoisomerase IV
DNA
Quinolone
DNA synthesis and
cell growth blocked
Bacteriostatic
Mechanism
Bactericidal
Mechanism
Cell death
Cell death

The result of both models is the release of double-stranded DNA breaks during the denaturation of the topoisomerase, perhaps during an aborted repair attempt (24, 28, 58, 70, 75). The release of the DNA ends are believed to stimulate bacterial apoptosis (43).

iii. Target Preference

The majority of fluoroquinolones are reported to preferentially target either DNA gyrase or topoisomerase IV although all fluoroquinolones can bind both enzymes to varying degrees (95, 96, 98, 109, 130). In general, DNA gyrase is the primary target in Gram-negative organisms and topoisomerase IV is the primary target in Gram-positive organisms (47, 111). In *S. pneumoniae*, different fluoroquinolones preferentially bind to either DNA gyrase or topoisomerase IV. Topoisomerase IV is the primary target of ciprofloxacin and levofloxacin and DNA gyrase is the primary target of gatifloxacin and moxifloxacin (47, 58, 98, 111, 128).

In order to limit the emergence of resistance, it has been the aspiration of drug discovery programs to identify fluoroquinolones that possess dual-activity. Dual-acting fluoroquinolones demonstrate comparable activity against both DNA gyrase and topoisomerase IV. An organism would have to generate point mutations in both DNA gyrase and topoisomerase IV in order to become resistant to such a fluoroquinolone, as single point mutations in one target alone would not yield clinically relevant resistance i.e. organisms whose MICs increased beyond breakpoint levels (84, 109). As double mutations are a rare genetic event occurring at a frequency of 10^{-14} for fluoroquinolones in *S. pneumoniae* (18), the preferential use of fluoroquinolones with dual-activity could limit the development of fluoroquinolone resistance in *S. pneumoniae*. Although gemifloxacin is sometimes reported as dual-active (58), others have reported it as

preferentially binding to either DNA gyrase or topoisomerase IV (85, 122). A dual-active, safe fluoroquinolone has yet to be discovered (119).

d. Mechanisms of Resistance

Resistance to fluoroquinolones in *S. pneumoniae* is mediated by at least three mechanisms: spontaneous chromosomal mutations in the quinolone resistance-determining regions (QRDRs) of DNA gyrase and topoisomerase IV and active efflux.

i. Chromosomal mutations

Fluoroquinolone resistance in *S. pneumoniae* most frequently results from point mutations causing amino acid substitutions in the QRDRs of DNA gyrase and topoisomerase IV. Resistance arises from the stepwise accumulation of mutations (109). Mutations have been observed in *gyrA*, *gyrB*, *parC*, and *parE*; however, amino acid substitutions in GyrA and ParC are the most common causes of fluoroquinolone resistance. In fluoroquinolone-resistant clinical *S. pneumoniae* isolates with single QRDR substitutions, the substitution is generally observed in ParC (22, 23, 25, 36, 66, 107, 137). GyrA substitutions are most commonly observed in conjunction with a ParC substitution resulting in highly fluoroquinolone-resistant *S. pneumoniae* isolates (22, 23, 25, 36, 66, 107, 137). The GyrA and ParC substitutions observed in *S. pneumoniae* are similar to the substitutions mediating fluoroquinolone resistance in various organisms including *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and many others (101, 111).

The QRDR was identified in *E. coli* and encompasses the region from amino acid 67 to 106 in GyrA (132). These amino acids are near the enzyme's active site, Tyr122, and substitutions in this region may diminish the binding of the fluoroquinolones to the

DNA-enzyme complex (93, 96). The specific amino acids in GyrA that are commonly altered in fluoroquinolone-resistant *S. pneumoniae* isolates are Ser81 and Glu85 (92, 96). Ser81 is generally substituted with a phenylalanine or tyrosine (89, 92). Recently, a substitution of isoleucine at position 81 was reported (72). Glu85 is commonly substituted to a glycine or lysine (92). Substitutions of Glu85 are associated with isolates displaying high-level fluoroquinolone resistance (11). Amino acid substitutions of Ser81 and Glu85 may alter the quinolone binding site structure of the DNA gyrase-DNA complex resulting in a reduced binding affinity and fluoroquinolone resistance.

As there is a high degree of sequence homology between the QRDRs of GyrA and ParC, similar resistance-causing substitutions are observed in both enzymes. The ParC amino acid substitutions commonly associated with fluoroquinolone resistance are Ser79 and Asp83 (89, 93). The common substitutions of Ser79 are phenylalanine and tyrosine (93). Other substitutions reported for Ser79 include alanine, arginine, and leucine (22, 72). Asp83 can be substituted by alanine, asparagine, glycine, histidine, tyrosine or valine (11, 89). Two novel substitutions, Ser80Phe and Gln90His, have recently been reported as contributing to fluoroquinolone resistance (22). Asp78Ala/Asn has been infrequently observed (23, 38, 66). Ser52Gly and Lys137Asn are other commonly observed ParC substitutions, but these do not appear to contribute to fluoroquinolone resistance (11, 66, 72, 106).

Additional GyrA and ParC substitutions that are believed to have been incorporated from the viridans group streptococci are increasingly reported in the literature (22, 103). These substitutions include Ser114Gly in GyrA and Asn91Asp in

ParC (22). The impact of these substitutions on the development of fluoroquinolone resistance in *S. pneumoniae* remains to be seen.

Substitutions have also been observed in GyrB and ParE, although they rarely result in fluoroquinolone resistance. A substitution of Glu474Lys has been reported in GyrB of *S. pneumoniae* (101). ParE substitutions occur more commonly than GyrB substitutions in fluoroquinolone-resistant *S. pneumoniae*. ParE substitutions that have been associated with fluoroquinolone resistance include Asp435Asn and Pro454Ser (94, 97). Other reported ParE substitutions include Ala325Val, Arg447Ser, and Ile460Val, but their significance in resistance generation has yet to be determined (11, 22, 89). Generally, GyrB and ParE substitutions are only observed in fluoroquinolone-resistant *S. pneumoniae* clinical isolates that also possess GyrA and/or ParC substitutions making the role of the GyrB and ParE substitutions unclear. The amino acid substitutions observed in GyrA, GyrB, ParC, and ParE that have been associated with fluoroquinolone resistance in *S. pneumoniae* are summarized in table 1.1.

Table 1.1 Amino acid substitutions associated with fluoroquinolone resistance in *S. pneumoniae* in GyrA, GyrB, ParC and ParE

GyrA	GyrB	ParC	ParE
Ser81 to Phe/Tyr Glu85 to Gly/Lys	Glu474 to Lys	Asp78 to Ala/Asn Ser79 to Ala/Arg/Leu/Phe/Tyr Ser80 to Phe Asp83 to Ala/Asn/Gly/His/Tyr/Val Gln90 to His	Asp435 to Asn Pro454 to Ser

ii. Efflux

Fluoroquinolone efflux has been reported in various Gram-positive organisms, including *Bacillus subtilus*, *S. aureus*, *S. pneumoniae*, the viridans group streptococci,

and enterococci (104). The fluoroquinolone efflux systems identified in Gram-positive organisms are able to pump out numerous unrelated compounds such as antimicrobials, dyes, and lipophilic cations (104). This has lead to the conclusion that these multidrug efflux pumps are an intrinsic part of the organism's functioning, which act independently of antimicrobial efflux (104). The efflux pumps NorA and Bmr in *S. aureus* and *B. subtilis*, respectively, have been well-characterized. Reserpine, a plant alkaloid, was first shown to block Bmr-mediated drug resistance and is used to identify NorA-type efflux pumps in Gram-positive organisms (104).

The involvement of a multi-drug efflux pump in fluoroquinolone resistance in *S. pneumoniae* was first indicated by increased susceptibility to fluoroquinolones and decreased resistant mutant selection in the presence of reserpine (8, 83). Gill *et al.* were the first to identify a putative efflux pump of fluoroquinolones in *S. pneumoniae* that showed homology to NorA and Bmr (50). The pump had 24% sequence identity to each of NorA and Bmr (50). The pump is referred to as PmrA (pneumococcal multidrug resistance protein). Like NorA and Bmr, PmrA is a major facilitator superfamily pump of the 12-transmembrane-segment class (50, 104). Similarly to NorA, PmrA pumps a limited range of fluoroquinolones (104). In *S. pneumoniae*, fluoroquinolone efflux of hydrophilic molecules like ciprofloxacin and norfloxacin occurs more readily than efflux of hydrophobic molecules like the respiratory fluoroquinolones (134).

Recently, the role of PmrA in fluoroquinolone resistance in *S. pneumoniae* has been questioned. *pmrA* had been identified in all *S. pneumoniae* isolates regardless of their susceptibility to the fluoroquinolones so it had been hypothesized that the resistance phenotype resulted from an increase in the expression of PmrA (50). Expression levels

of PmrA were not found to correlate with the efflux phenotype (102). Additionally, the inactivation of PmrA in *S. pneumoniae* isolates did not affect their fluoroquinolone susceptibilities (99). It has thus been suggested that *S. pneumoniae* have other efflux pumps that are inhibited by reserpine and involved in fluoroquinolone resistance (83, 99, 102). Genome sequencing has revealed a large number of potential efflux pump genes of which approximately 70 do not have an assigned function (19). A great deal of research is required to determine both the role of efflux in fluoroquinolone resistance and the actual efflux pumps involved.

e. Mutant Prevention Concentration

Implementing new dosing strategies for fluoroquinolones is one technique that has been suggested as a potential method to limit the emergence of resistance. In resistance development, resistant mutants are randomly generated during replication and are selected within the bacterial population by antimicrobial therapy (139). Blocking the original generation of mutants is not currently possible so Drlica and Zhao have proposed a method to block the selective enrichment of resistant mutants (139). The suggestion is based on the notion that there is a concentration range for every fluoroquinolone-pathogen combination that selects resistant mutants (42). This range has been termed the mutant selection window (MSW) (42, 139). The mutants selected within the MSW may have target mutations or become efflux-positive (40, 140). The limits of the MSW are the concentration that inhibits the majority of susceptible growth, the MIC, and the concentration that inhibits all organisms containing a single resistance-causing mutation (42, 139). The bacterial cells must develop two or more resistance-causing mutations in order to grow in the presence of fluoroquinolone concentrations greater than the upper

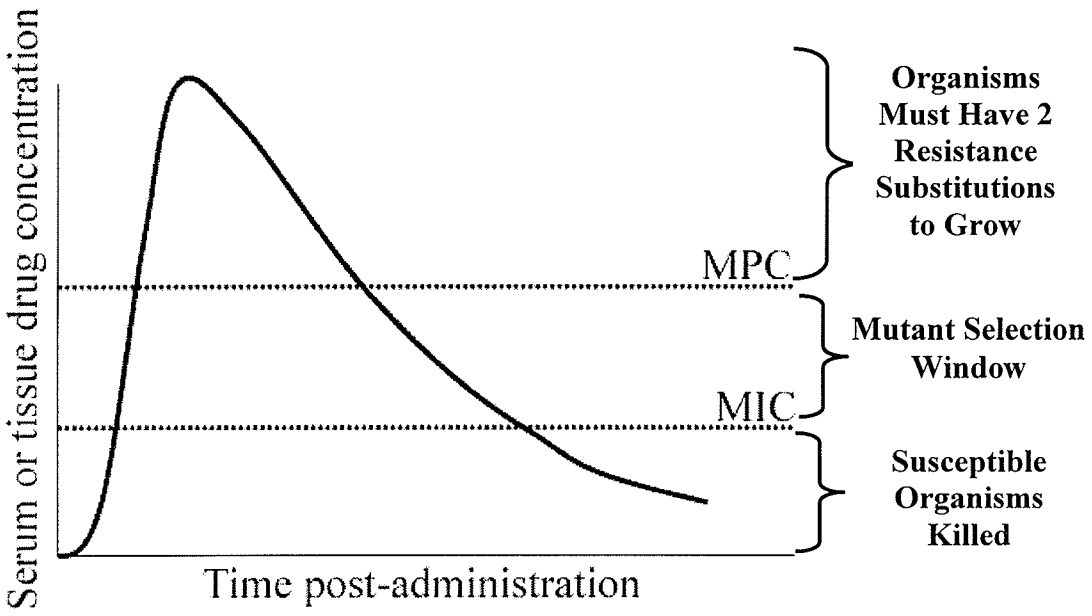
limit of the MSW (18, 41, 42, 128, 139). This antimicrobial level is referred to as the mutant prevention concentration (MPC) (18, 41, 42, 139). In this way, the MPC is the MIC of the least-susceptible single-step mutant (18). The MPC concept is diagrammatically portrayed in figure 1.5. Dosing above the MPC has been suggested as a method by which the selection of mutants during antimicrobial treatment could be minimized (41). The MSW and MPC concepts for the fluoroquinolones have recently been supported by *in vivo* testing (34, 45, 141).

Many aspects of the MSW concept affect fluoroquinolone therapy. The size of the MSW is important as it is the range of antimicrobial concentrations that best selects resistant mutants. The most important parameters are whether the antimicrobial concentration at the site of infection is above the MPC and the amount of time it remains above the MPC (61). In order to be clinically relevant, the MPC must be below the serum and tissue concentrations attainable upon the administration of safe doses of antimicrobials (41).

The MPC has been experimentally defined as the drug concentration that prohibits the growth of mutants from a susceptible population of more than 10^{10} cells (18, 41, 117). 10^{10} cells are plated in the presence of doubling-dilutions of a fluoroquinolone and the concentration at which no growth is observed is recorded as the MPC. A concentration of 10^{10} cells is used to detect mutational frequencies of 10^{-7} to 10^{-9}, the mutational frequency of *S. pneumoniae* for fluoroquinolones (41), and to mimic the typical bacterial load and population heterogeneity at a site of infection (128). Based on a mutational frequency of 10^{-7}, an infection of more than 10^{14} *S. pneumoniae* would be

Figure 1.5 **Diagrammatic representation of the Mutant Prevention Concentration Theory (Adapted from (42)).**

Fig. 1.5


Serum or tissue drug concentration
Time post-administration
MPC
MIC
Organisms Must Have 2 Resistance Substitutions to Grow
Mutant Selection Window
Susceptible Organisms Killed

required in order for two simultaneous fluoroquinolone resistance-causing mutations to occur (18, 42). Dosing at the MPC would limit the selection of resistant mutants.

In addition to a new dosing strategy, the MPC has been suggested as an *in vitro* measure of a fluoroquinolone's potency as the specific structure of each fluoroquinolone affects the MPC (18, 41, 128). The MPC may provide a more effective assessment of an antimicrobial's ability to prevent the selection of resistant mutants than the MIC as the inoculum used in the MPC determination emulates the bacterial load of common *S. pneumoniae* infections (61).

The MPC concept has generally been applied to *S. pneumoniae, S. aureus,* and *Mycobacterium* species with fluoroquinolones; however, the concept has also been applied to a variety of organisms and antimicrobials including *E. coli, Acinetobacter baumannii*, *Citrobacter freundii*, *Enterobacter cloacae*, *Klebsiella pneumonia*, *P. aeruginosa*, and *Stenotrophomonas maltophilia* with fluoroquinolones, macrolides, tobramycin, chloramphenicol, rifampicin, penicillin, and vancomycin (3, 4, 16, 18, 34, 40, 45, 138, 141). It is essential that the MPC concept only be applied to organism-antimicrobial combinations in which the resistance mechanisms tested match those observed clinically (120). The resistance mechanisms observed with the fluoroquinolone MPC studies in *S. pneumoniae*, chromosomal mutations, are the same as those observed in clinical isolates. Thus, the fluoroquinolone MPCs for *S. pneumoniae* have the potential to restrict resistance development if applied therapeutically.

3. Rationale

Few novel antimicrobials are being developed by the pharmaceutical industry. Accordingly, it is essential that available antimicrobials, particularly those as active as the fluoroquinolones, be preserved for the future. As fluoroquinolone use increases in Canada, an increase in resistance will likely follow. Fluoroquinolone resistance in *S. pneumoniae* remains low in Canada; however, recent increases are disconcerting. An improved understanding of the development of resistance and appropriate usage will be necessary in order to maintain the efficacy of these agents.

Surveillance and molecular analysis of the fluoroquinolone-resistant *S. pneumoniae* in Canada are essential in order to identify the mechanisms of the increase in resistance and evaluate methods aimed at limiting resistance development.

4. Thesis Objectives

The hypothesis of this research is continued and increasing usage of fluoroquinolones in Canada will selectively genetically alter *S. pneumoniae* such that they will become increasingly fluoroquinolone resistant over time, but novel therapeutic approaches may limit this increase in resistance. Accordingly, it is essential that the fluoroquinolones be fully characterized for activity, resistance development, and mechanisms to prevent the selection of resistant isolates.

In order to address this hypothesis, three important questions arise.

1. How have fluoroquinolone resistant isolates evolved throughout the past 7 years in Canada, during which time isolates have been collected for analysis? As part of this broad question, many specific questions must be investigated. What

mutations are present in these resistant isolates? Do mutations outside the traditional QRDR affect fluoroquinolone resistance? What is the impact of efflux? Is fluoroquinolone resistance clonally spreading in Canada? Are particular serotypes associated with fluoroquinolone resistance? To determine how fluoroquinolone resistance has evolved over time in Canada, ciprofloxacin-resistant *S. pneumoniae* isolates collected over 7 years from across Canada were molecularly characterized by resistance mechanisms (chromosomal mutations and efflux), serotype and pulsed-field gel electrophoresis pattern.

2. Are resistance-associated mutations accumulating in fluoroquinolone-susceptible isolates which may be undetected by standard methodologies and subsequently to further treatment with a fluoroquinolone have the potential to become highly fluoroquinolone resistant? In order to investigate this question, the prevalence of fluoroquinolone resistance-associated mutations in fluoroquinolone-susceptible *S. pneumoniae* isolates was determined at two time points (pre-1997 and 2003). Microbiological resistant breakpoints for the fluoroquinolones were determined based on the likelihood of chromosomal mutations at particular MICs. Microbiological breakpoints identify isolates with chromosomal mutations as resistant, which would remain undetected using current breakpoint systems. Accordingly, the microbiological breakpoints would permit a more sensitive means to conduct surveillance of genetic alterations resulting in fluoroquinolone resistance.
3. Could novel dosing strategies like the MPC limit the emergence of resistance? *S. pneumoniae* isolates with a variety of genetic backgrounds, relevant to

fluoroquinolone resistance, are circulating and infecting people worldwide. If novel dosing strategies such as the MPC are to be employed to limit the selection of resistance, the affect of the existing genetic backgrounds on the MPCs must be determined. Throughout this study, MPCs were evaluated for ciprofloxacin, gatifloxacin, gemifloxacin, levofloxacin, and moxifloxacin with *S. pneumoniae* isolates of known genetic background, related to fluoroquinolone resistance.

The surveillance of fluoroquinolone resistance in *S. pneumoniae* conducted throughout this study is necessary in order to monitor the resistance mechanisms as fluoroquinolone resistance increases in Canada and to provide a basis for the evaluation of novel strategies aimed at limiting resistance development.

B. MATERIALS AND METHODS

1. Bacterial Isolates

a. Isolate Selection

Over 9,000 clinical isolates of *S. pneumoniae* were submitted to or isolated by the Department of Clinical Microbiology, Health Sciences Centre, Winnipeg, Manitoba, Canada between August 1997 and December 2004 as part of an ongoing Canadian Respiratory Organism Susceptibility Study (CROSS) (136). Prior to 2003, the CROSS study year was from November 1st of one year to October 31st of the following year. The study years are accordingly listed as 1997/98, 1998/99, 1999/2000, 2000/01, and 2001/02 in the following text. Commencing in 2003, the CROSS study years followed the calendar year and are listed so throughout the thesis. CROSS includes *S. pneumoniae* isolates from 25 health care centres in 9 of the 10 Canadian provinces (136).

All ciprofloxacin-resistant (MIC $\geq$ 4 μg/mL) isolates received between 1997 and 2004 (156) were molecularly characterized throughout this study. The ciprofloxacin-resistant isolates were obtained from 22 health care centres distributed across 9 Canadian provinces: Alberta, British Columbia, Manitoba, New Brunswick, Nova Scotia, Ontario, Prince Edward Island, Quebec, and Saskatchewan. The isolates were primarily sputum samples (90%), as well as endotracheal tube, lung aspirate and tracheal specimens.

Of these isolates, 54 were randomly selected for full gene sequencing analysis of *gyrA*, *parC* and *parE* to identify any non-QRDR mutations associated with ciprofloxacin resistance. Isolates were selected such that various genotypes were included: no QRDR substitutions in GyrA or ParC, single GyrA QRDR substitutions, single ParC QRDR substitutions, and QRDR substitutions in both GyrA and ParC.

Additionally, over 900 ciprofloxacin-susceptible isolates collected as part of CROSS and related studies between November 1995 and December 2003 were studied in the analysis of fluoroquinolone resistance-associated amino acid substitutions in Canadian clinical fluoroquinolone-susceptible *S. pneumoniae* isolates. The isolates were collected from 9 Canadian provinces: Alberta, British Columbia, Manitoba, New Brunswick, Nova Scotia, Ontario, Prince Edward Island, Quebec, and Saskatchewan. The isolates were primarily sputum samples (75%), as well as tracheal specimens, bronchial specimens, and other respiratory samples.

For the mutant prevention concentration studies, 3 fluoroquinolone-susceptible isolates collected as part of CROSS (stock numbers: 2587, 2663, 2670) and 2 isolates (984 and 1146) received from Brueggemann *et al.* (23) from a multi-centre surveillance study in the United States were included. The other 6 isolates were ciprofloxacin-resistant isolates evaluated in the molecular characterization study.

Two reference strains of *S. pneumoniae* were used throughout these studies. *S. pneumoniae* ATCC® 49619 was included as a control for antimicrobial susceptibility testing, polymerase chain reaction (PCR) amplification methods, and pulsed-field gel electrophoresis (PFGE) techniques. *S. pneumoniae* R6 (ATCC® BAA-255) was used as the reference for the sequence alignments. Additionally, it was used as the recipient strain in the transformation experiments.

b. Isolate Identification

The identity of each *S. pneumoniae* isolate was confirmed by Gram stain, colony morphology, α-hemolytic growth pattern on Trypticase soy agar plates with 5% sheep blood (SBA), and the results of Bile Solubility and Optochin Tests as recommended in

the Manual of Clinical Microbiology (108). Subsequent to identification, *S. pneumoniae* isolates were inoculated into skim milk and stored at -80°C.

2. Antimicrobial Preparation

Antimicrobials were obtained as laboratory grade powders from their respective manufacturers: ciprofloxacin (Bayer Canada Inc.), gatifloxacin (Bristol-Myers Squibb Canada), gemifloxacin (GlaxoSmith-Kline), levofloxacin (Janssen-Ortho Inc.), and moxifloxacin (Bayer Canada Inc.). Antimicrobials were reconstituted and stored according to the CLSI guidelines (1). The activity of the antimicrobials was confirmed using *S. pneumoniae* ATCC® 49619, *Staphylococcus aureus* ATCC® 29213, and *Pseudomonas aeruginosa* ATCC® 27853.

3. Determination of MICs

a. Broth Microdilution

Following two subcultures from frozen stock on SBA, the antimicrobial susceptibilities of the *S. pneumoniae* isolates were tested by the CLSI M7-A6 broth microdilution method as part of CROSS (1). Custom-designed 96-well microtiter plates containing doubling antimicrobial dilutions in 100 μL of cation-adjusted Mueller-Hinton broth plus 2 – 5% vol/vol lysed horse blood were made in-house. The tested antimicrobials include: penicillin, amoxicillin-clavulanate, cefuroxime, cefprozil, cefixime, cefaclor, cefotaxime, ceftriaxone, imipenem, meropenem, erythromycin, azithromycin, clarithromycin, clindamycin, tetracycline, doxycycline, chloramphenicol, trimethoprim-sulfamethoxazole, vancomycin, quinupristin-dalfopristin, ciprofloxacin,

levofloxacin, gatifloxacin, gemifloxacin, moxifloxacin, linezolid, telithromycin, ertapenem, and tigecycline (136). Bacterial suspensions were prepared in sterilized water and adjusted to a McFarland turbidity standard of 0.5 (equivalent to 1-2 x 10^8 CFU/mL (1)). Panels were inoculated with a final bacterial inoculum of 5 x 10^5 CFU/mL and incubated for 22 - 24 h at 35°C in ambient air (2, 136). The MICs, defined as the lowest concentration of an antimicrobial that completely inhibited visible growth, were recorded following incubation. The MICs of the ciprofloxacin-resistant isolates were conducted at least in triplicate on separate days to ensure reproducibility. Colony counts were performed to confirm inocula.

b. E-test

E-tests were conducted according to the manufacturer's instructional document (AB Biodisk, Solna, Sweden). A 0.5 McFarland standard was prepared for each organism, streaked onto Mueller Hinton agar containing 5% lysed sheep blood, and allowed to dry prior to the application of the E-test strips (62). E-test strips were aseptically applied to the inoculated surface and plates were incubated for 20 – 24 hours at 35°C in 5% CO_2. Ciprofloxacin, gatifloxacin, gemifloxacin, levofloxacin, and moxifloxacin MICs were read at the point of intersection between the ellipse of growth inhibition and the MIC scale on the E-test strip.

4. Sequencing

a. DNA Isolation

Genomic DNA was isolated from cultures of *S. pneumoniae* grown overnight on SBA. A small loopful of bacteria was emulsified in lysis solution as described by

Ubukata *et al.* (127). Bacterial cell lysis was accomplished by incubation of cell suspensions at 60°C for 10 minutes and then 94°C for 5 minutes in a Perkin-Elmer GeneAmp® PCR System 9700. The resultant lysates were used as DNA templates for the PCR described in sections 4b and c.

b. Amplification of the Quinolone Resistance-Determining Regions of *gyrA* and *parC*

For amplification of the QRDRs of *gyrA* and *parC*, primers and conditions previously described by Morrissey *et al.* were used in the PCR (84, 137). The primers are listed in table 2.1. Amplification of the QRDRs of *gyrA* and *parC* was conducted using a Perkin-Elmer GeneAmp® PCR System 9700.

Table 2.1 Primers used for amplification of QRDRs of *gyrA* and *parC* (84)

Primer	Sequence (5'-3')	Nucleotide Position
GyrA forward	TAAAAAACTTTGTCACGAATATGCC	130-105 bp upstream of start
GyrA reverse	AACGATACGCTCACGACCAGT	750-771
ParC forward	AAACCTACTCTACATTCTTTGAAAGGAG	134-106bp upstream of start
ParC reverse	CAGTTGGGTGGTCAATCATGTAAA	571-594

bp, base pair

c. Amplification of *gyrA*, *parC*, and *parE*

Amplification of *gyrA*, *parC*, and *parE* from *S. pneumoniae* isolates was conducted by PCR using 2 previously described primers (84) and 4 primers designed throughout this project. The primers used for the amplification of *gyrA*, *parC*, and *parE* are shown in table 2.2. The amplification reactions consisted of 5 μL of 15 mM $MgCl_2$-10X PCR buffer, 1mM of dNTPs, 10 μM of each primer, 1 mM $MgCl_2$, 2.5 U of Taq

DNA polymerase, 5 μL of the DNA template, and sterile water to a final volume of 50 μL. Thermocycler conditions for *parC* and *parE* were 30 cycles of 94°C for 5 minutes, 94°C for 30 seconds, 50°C for 1 minute, and 72°C for 3 minutes with a final extension of 7 minutes at 72°C. The thermocycler conditions for *gyrA* were 30 cycles of 94°C for 5 minutes, 94°C for 30 seconds, 57°C for 45 seconds, 72°C for 2 minutes and 15 seconds, and a final extension of 7 minutes at 72°C. Reactions were conducted with a Perkin-Elmer GeneAmp® PCR System 9700.

Table 2.2 Primers used for amplification of *gyrA*, *parC*, and *parE*

Primer	Sequence (5'-3')	Nucleotide Position	Source
GyrA forward	TAAAAAACTTTGTCACGAATATGCC	130-105 bp upstream of start	(84)
GyrA reverse - long	GAGACATTATGCTTCACCTTCTG	2453-2475	This study
ParC forward	AAACCTACTCTACATTCTTTGAAAGGAG	134-106bp upstream of start	(84)
ParC reverse - long	CCACTCCTTATTCTAAAAACC	2579-2599	This study
ParE forward	CCAGATGGAATCGAACCC	191-175 bp upstream of start	This study
ParE reverse	CCTTTCAAAGAATGTAGAGTAGG	2234-2256	This study

bp, base pair

d. Agarose Gel Electrophoresis

The amplified DNA fragments were analyzed by electrophoresis through agarose gels (2% for QRDR products and 1% for full gene products) made with 0.5X Tris-

Borate-EDTA and containing ethidium bromide. Gels were run for 1 hour at 100V and bands were visualized under UV transillumination. A 123 bp ladder was used as a molecular weight standard for the *gyrA* and *parC* QRDR products. For the *gyrA*, *parC*, and *parE* gene products, a 1 Kb DNA ladder was used as a molecular weight standard.

e. Purification and Quantitation of DNA Template for Sequencing

PCR products were purified using Microcon® YM100 centrifugal filter units (Millipore, Bedford, MA) according to the manufacturer's instructions. The purified PCR products were eluted from the columns with Tris (10mM)-EDTA (1mM) pH 8.0 buffer. DNA recovery was verified by gel electrophoresis as described in section 5 d. The purified PCR products were quantitated 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 of 1 is at a concentration of 50 μg/mL in a 10 mm pathlength cell.

f. Sequencing Reaction

Sequencing reactions were performed with the ABI PRISM® BigDye Terminator Cycle Sequencing Ready Reaction Kit using previously described conditions (137). Each sequencing reaction was made with 100 ng of purified PCR product. Sequencing reactions were carried out in forward and reverse directions with the primers listed in table 2.3. The primers used to sequence the QRDRs of *gyrA* and *parC* have been previously described (84). The primers designed in 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.

Table 2.3 **Primers used for sequencing of *gyrA*, *parC*, and *parE* and the QRDRs of *gyrA* and *parC***

Primer	Sequence (5'-3')	Nucleotide Position	Source
QRDRs of *gyrA* and *parC*			
GyrA SF	CGTTTTAGTGGTTTAGAGGC	85-66 bp upstream of start	(84)
GyrA SR	GACCAACTTCACTGCATC	567-585	(84)
ParC SF	CGCCCTAGATACTGTGTGA	98-80 bp upstream of start	(84)
ParC SR	AAATCCCAGTCGAACCAT	493-510	(84)
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

bp, base pair

g. Purification of Sequencing Products by Ethanol/Sodium Acetate Precipitation

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 (137). Purified sequencing products were reconstituted in 15 μL of Template Suppression Reagent or Formamide for analysis on the ABI PRISM® 310 or 3100 Genetic Analyzers (Applied Biosystems, Foster City, CA), respectively.

h. Sequence Analysis

Sequence analysis was conducted on the ABI PRISM® 310 or 3100 Genetic Analyzers (Applied Biosystems, Foster City, CA) in accordance with the manufacturer's instructions.

i. Sequence Alignment

Utilizing Lasergene's Seqman II module (DNAStar Inc., Madison, WI) or ABI PRISM® SeqScape® Software (Applied Biosystems, Foster City, CA) forward and reverse sequences for each isolate were combined into one specimen. The specimens were aligned and compared to the published sequence of *S. pneumoniae* R6 using Lasergene's Megalign module (DNAStar Inc., Madison, WI) or ABI PRISM® SeqScape® Software (Applied Biosystems, Foster City, CA). Amino acid substitutions in the sequenced isolates, as compared to R6, were recorded.

5. Reserpine-inhibited Efflux Identification

The following reserpine efflux identification protocol was kindly provided by Dr. D. Bast (Mt. Sinai Hospital, Toronto, ON) (11).

a. Creation of Ciprofloxacin Agar Plates with and without Reserpine

Mueller-Hinton agar plates plus 5% sheep blood were made with doubling dilutions of ciprofloxacin with and without reserpine (10 μg/mL). The agar plates were made with a ciprofloxacin concentration range from 2 dilutions greater than the highest expected MIC, based on the microbroth dilution MICs, and 4 dilutions lower than the lowest MIC. This range takes into account that some isolates survive at slightly higher antimicrobial concentrations on agar than the microbroth dilution MICs suggest and that there could be an 8-fold MIC reduction in the presence of reserpine. The agar plates containing reserpine were made the same day as the efflux study was conducted as reserpine is unstable.

b. Agar Dilution MICs

Bacterial suspensions were adjusted to a 0.5 McFarland standard in Mueller-Hinton broth for each isolate, which had been sub-cultured twice from frozen stock. Isolates were diluted and loaded into the wells of a Steer's replicater such that the final inoculum delivered by the replicater's pins was 10^4 CFU. The ciprofloxacin plates, with and without reserpine, were inoculated in increasing order of antimicrobial 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. *S. pneumoniae* ATCC® 49619, *E. faecalis* ATCC® 29212, *P. aeruginosa* ATCC® 27853, and *S. aureus* ATCC® 29213 and a ciprofloxacin-resistant,

efflux-positive laboratory *S. pneumoniae* isolate, CA813, were included as controls. Plates were incubated at 35°C for 20-24 hours, one set in ambient air for *E. faecalis, P. aeruginosa, and S. aureus* and one set in CO_2 for the *S. pneumoniae* controls and study isolates. All isolates were tested in duplicate to ensure reproducibility.

c. Efflux Identification

Following the incubation period, the MICs were recorded for each isolate in the presence and absence of reserpine. Isolates demonstrating a fourfold or greater reduction in MIC in the presence of reserpine were considered positive for reserpine-sensitive efflux (11).

6. Pulsed-Field Gel Electrophoresis

a. Pulsed-Field Gel Electrophoresis of Macrorestriction Fragments

Preparations of genomic DNA, *Sma*I digestions, and electrophoresis conditions for PFGE were performed as previously described by Louie *et. al* (78). Gels were stained for 50 min with 20 μL of Sybr Green in 150 mL of Tris-HCl (10 mM)-EDTA (1mM), destained with distilled water, and examined under UV transillumination (137).

b. Pattern Analysis

PFGE profiles were scanned and digitized with the Gel Doc 1000 System (Bio-Rad Laboratories, Hercules, CA) and analyzed using BioNumerics™ version 3.5 (Applied Maths, Austin, Texas). A dendrogram was calculated by the unweighted pair group method with arithmetic averages (Band Tolerance: 1% and Dice Coefficient 1%). For the purpose of this study, isolates were defined as genetically indistinguishable, possibly related, or genetically unrelated if their PFGE profiles differed by 0, 1-3, or ≥4

bands, respectively (90). Isolates with more than 80% similarity on the dendrogram have been correlated with a 3 or less band difference, i.e. genetically related.

7. Serotyping

Isolates collected prior to 2001 were serotyped by the National Centre for Streptococcus (Edmonton, Alberta). Subsequent to 2001, isolates were serotyped in-house on the basis of capsular polysaccharide antigens by the Quellung reaction following standard methodology (6). Type-specific antisera were obtained from the Statens Seruminstitut (Copenhagen, Denmark). Global clones previously described by the Pneumococcal Molecular Epidemiology Network (www.sph.emory.edu/PMEN) were routinely serotyped as controls.

8. Single-step Fluoroquinolone-resistant Mutants

a. Isolate Selection

Nine clinical *S. pneumoniae* isolates collected as part of CROSS (136) and 2 isolates received from Brueggemann *et al.* (23) were selected such that all phenotypes/genotypes known to be pertinent to fluoroquinolone resistance development were included. Three ciprofloxacin-susceptible (wild-type ParC/GyrA and efflux-negative) isolates: 2587, 2663, and 2670, 2 ciprofloxacin-resistant (wild-type ParC/GyrA and efflux-positive) isolates: 15017 and 16072, 2 ciprofloxacin-resistant (ParC: Ser79Phe substitution, wild-type GyrA and efflux-negative) isolates: 4610 and 14744, 2 ciprofloxacin-resistant (GyrA: Ser81Phe substitution, wild-type ParC and efflux-negative) isolates: 1146 and 23786, and 2 ciprofloxacin-resistant (ParC: Ser79Phe, GyrA:

Ser81Phe, and efflux-negative) isolates: 984 and 17012 were used to determine the mutant prevention concentrations of ciprofloxacin, gatifloxacin, gemifloxacin, levofloxacin, and moxifloxacin (122).

b. Selection of Single-step Fluoroquinolone-Resistant Mutants

The isolates were grown overnight on SBA at 35°C in a CO_2 incubator. The overnight growth was swabbed into Mueller-Hinton broth with 2% lysed horse blood and incubated for 45 minutes at 35°C in ambient air in order to achieve an inoculum near 10^{10} CFU/mL (122). Spontaneous single-step *S. pneumoniae* mutants were obtained by plating the inoculums on Mueller-Hinton agar plates containing 5% sheep blood and 1x, 2x, 4x, 8x or 16x MIC of ciprofloxacin, gatifloxacin, gemifloxacin, levofloxacin or moxifloxacin (122, 124). Additionally, the inoculums were plated onto drug-free SBA to obtain a total CFU/mL. The antimicrobial-containing plates were incubated in a CO_2 enriched environment at 35°C for 48-72 hours and the antimicrobial-free plates were incubated under the same conditions for 24 hours.

c. Mutational Frequency Calculation

Colony counts were conducted after the appropriate incubation period. The mutational frequencies were calculated as the ratio of colonies grown on antimicrobial-containing plates to colonies formed on drug-free plates (92, 122, 124).

d. Mutant Prevention Concentration Determination

The mutant prevention concentration for each drug-isolate combination was defined as the lowest fluoroquinolone concentration that prevented growth of resistant mutants (42) while the mutant prevention MIC (MP_{MIC}) was the multiple of MIC

corresponding to the MPC (122). All resulting mutants were stocked in skim milk and stored at -80°C for further study.

e. Resistance Mechanism Identification of Mutants

i. Sequencing of Quinolone Resistance Determining Regions in *gyrA* and *parC*

The single-step fluoroquinolone-resistant mutants were lysed, amplified, and the QRDRs of *gyrA* and *parC* were sequenced as described in sections 4 a, b, and d – i (122).

ii. Reserpine-Inhibited Efflux

The single-step fluoroquinolone-resistant mutants were tested for ciprofloxacin efflux by the agar dilution method in the presence and absence of reserpine as described in sections 5 a – c (122).

9. Stability of Fluoroquinolone Resistance Mechanisms

a. Selection of Clinical Isolates and Laboratory Mutants

Clinical isolates were selected such that various phenotypes/genotypes, including fluoroquinolone-susceptible, efflux-positive, ParC substitution, efflux-positive & ParC substitution, GyrA substitution, and substitutions in both ParC and GyrA, were evaluated. The most resistant mutant generated with each fluoroquinolone from every originating genotype was selected. Additionally, mutants with one-fold lower level of resistance to levofloxacin and moxifloxacin were studied when possible (e.g. mutants selected on levofloxacin 4x and 8x, if 8x was the highest concentration on which growth was observed). For the mutants created from the originating isolate containing a GyrA substitution, mutants with one-fold lower level of resistance to ciprofloxacin and

gemifloxacin were evaluated (118). In total, 17 clinical isolates and 26 lab-derived mutants were selected for this study (118).

b. Determination of MIC Stability by E-test

The 43 clinical isolates and laboratory mutants were sub-cultured every day for 20 days on antimicrobial-free SBA. The MICs of ciprofloxacin, gatifloxacin, levofloxacin and moxifloxacin were evaluated by E-test following the protocol described in section 3 b, every day for the first 10 days and again at day 20 (118). Gemifloxacin MICs could not be evaluated as gemifloxacin E-test strips were not commercially available at the time. Isolates were stocked in skim milk on days 5, 10, and 20 and stored at -80°C for further investigation. The study was repeated in order to ensure consistency.

c. Confirmation of Isolate Identity

Each isolate was confirmed to be genotypically identical prior to and following the twenty day study by PFGE in accordance with the protocol described in sections 6 a - d (90, 118).

d. Confirmation of Maintenance of Resistance Mechanism

The resistance mechanisms, amino acid substitutions in ParC or GyrA or efflux, identified in the isolates prior to the stability study were confirmed with the isolates for which an MIC decrease was observed overtime (118). The resistance mechanisms were tested following the methods described in sections 4 a, b, d - i and 5 a - c.

10. Transformations

a. Preparation of Transforming DNA

Chromosomal DNA was isolated, amplified, and purified as described in sections 4 a, b, d, and e. The transforming fragments were approximately 730 bp. Selected isolates and their pertinent amino acid substitutions, in parentheses, were as follows: 14744 (ParC: Ser79Phe), 45780 (ParC: Ser79Tyr), 14769 (ParC: Ser79Phe, Lys137Asn), and 19120 (ParC: Asp83Asn).

The following protocol for transformations in *S. pneumoniae* was kindly provided by Sarah Campbell (Queen Elizabeth II Health Sciences Centre, Halifax, NS).

b. Growth Curves

Following subculture twice from frozen stock, bacterial suspensions of *S. pneumoniae* R6 were made to a 3.0 McFarland standard in saline. The suspension was diluted 1:2 in Todd-Hewitt Broth with 5% Yeast Extract (THYB) and 0.2 mL of the dilution was inoculated into 20 mL of pre-warmed THYB to obtain a starting inoculum of approximately 4.5×10^6 CFU/mL. The culture was pre-incubated for 1 hour at 35°C in 5% CO_2. Samples were taken at various time points: 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, and 24 hours. Samples were serially diluted in saline. One hundred µL aliquots were plated onto SBA and incubated overnight at 35°C in 5% CO_2. The CFU/mL were determined the following day. Additionally at each time point, OD_{600} values were determined by spectrophotometry using the Ultrospec 2100 pro (Amersham Biosciences, Baie d'Urfe, QC).

c. Preparation of Pre-competent *S. pneumoniae*

Bacterial suspensions of *S. pneumoniae* R6 were made in THYB to obtain a starting concentration of approximately 4.5 x 10^6 CFU/mL in 20 mL of THYB as described in section 10b. Cultures were grown approximately 5.5 hours at 35°C in 5% CO_2 until the OD_{600} reached 0.2, a point in mid-log phase. Once cultures reached the desired OD_{600} value, cultures were chilled on ice, 2 mL of glycerol was added, and 0.5 mL aliquots were frozen at -80°C.

d. Transformation of Donor DNA into *S. pneumoniae*

An aliquot of pre-competent *S. pneumoniae* cells was thawed in a water bath at 37°C and diluted 1:100 in THYB (pH 6.0). After 1 hour of pre-incubation, a sample was taken, serially diluted, plated on SBA, and incubated overnight at 35°C in 5% CO_2 to confirm the starting concentration of cells was approximately 10^6 CFU/mL. The culture was incubated for about 2.5 hours at 35°C in 5% CO_2 until an OD_{600} of 0.04, a point in early log phase, was reached. Once cultures reached an OD_{600} of 0.04, they were supplemented with 0.2% bovine serum albumin and 0.01% $CaCl_2$. CSP-1 (100 ng), kindly provided by Dr. Don Morrison (Laboratory for Molecular Biology, Chicago, IL), was added to 1 mL aliquots of the supplemented culture. The transforming DNA (100 ng) was added, mixed, and the transformation mixture was incubated at 30°C for 2 hours and 15 minutes. *S. pneumoniae* R6 was subjected to the identical conditions described without the addition of transforming DNA to serve as a negative control.

e. Selection of Transformants

Following the incubation period, the transformation mixture was serially diluted in THYB and 100 µL samples were plated onto Mueller-Hinton agar with 5% sheep

blood and ciprofloxacin (1 μg/mL). The plates were incubated at 35°C in 5% CO_2 for 24 to 36 hours. Individual colonies were picked, plated on Mueller-Hinton agar with 5% sheep blood and ciprofloxacin (1 μg/mL), and grown overnight at 35°C in 5% CO_2 to obtain significant growth.

f. Confirmation of Transformation

To confirm the presence of the transformed mutations, transformants were lysed, and the QRDRs were amplified and sequenced as described in sections 4 a, b, and d-i.

g. MIC Testing of Transformants

The ciprofloxacin, gatifloxacin, gemifloxacin, levofloxacin, and moxifloxacin MICs of the transformants were tested by microbroth dilution as described in section 3 a.

11. Statistical Analysis

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

C. RESULTS

Part I: Characterization of Canadian Clinical Ciprofloxacin-resistant *S. pneumoniae* Isolates

a. Demographics

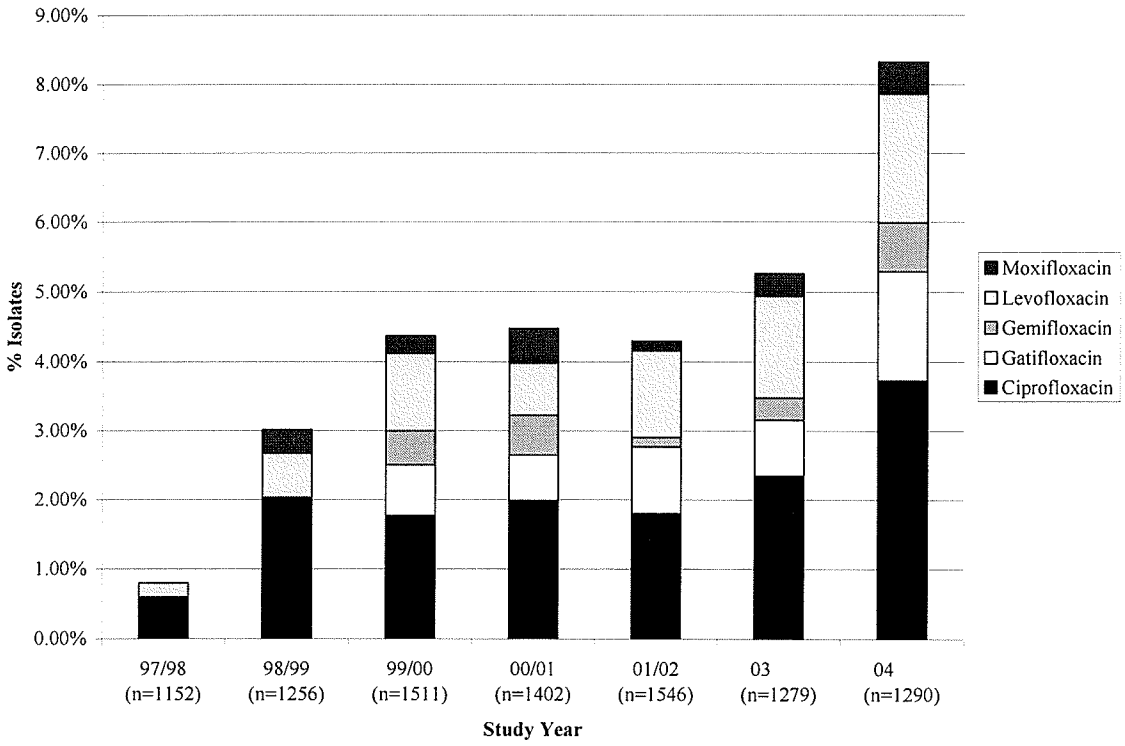
Between 1997 and 2004, over 9,000 *S. pneumoniae* isolates were collected as part of CROSS. The annual ciprofloxacin, gatifloxacin, gemifloxacin, levofloxacin, and moxifloxacin resistance rates of all isolates collected for CROSS are presented in figure 3.1. Statistical significance was calculated for the change in ciprofloxacin, gemifloxacin, and levofloxacin resistance rates observed between the first year the fluoroquinolone was tested in CROSS and 2004. Gatifloxacin and moxifloxacin resistance were tested for statistical significance from the year of introduction in Canada to 2004. Ciprofloxacin resistance increased from 0.6% in 1997/98 to 3.7% in 2004 (p=0.0001). Resistance increased from 0.7% to 1.6% for gatifloxacin between 2000/01 and 2004 (p=0.025). Gemifloxacin resistance increased from 0.5% to 0.7% between 1999/2000 and 2004 (p=0.46). Resistance to levofloxacin increased from 0.2% in 1997/98 to 1.9% in 2004 (p=0.0001). Moxifloxacin resistance was 0.5% in 2000/01 and 2004 (p=1.0).

One hundred and fifty-six of the *S. pneumoniae* isolates were determined to be ciprofloxacin-resistant (MIC $\geq$ 4 μg/mL) during the 7 years of collection. The demographics of these isolates are included in appendix A.

The majority of the ciprofloxacin-resistant isolates (65%) were isolated from males. Similarly, 61% of all CROSS *S. pneumoniae* isolates were isolated from males.

Figure 3.1 **Ciprofloxacin, gatifloxacin, gemifloxacin, levofloxacin, and moxifloxacin resistance rates for Canadian clinical *S. pneumoniae* isolates per year (1997/98 – 2004). Isolates were collected and MICs were tested by microbroth dilution as part of CROSS. The number of isolates collected each year is indicated in parentheses.**

Fig. 3.1


9.00%
8.00%
7.00%
6.00%
5.00%
4.00%
3.00%
2.00%
1.00%
0.00%
% Isolates
97/98 (n=1152)
98/99 (n=1256)
99/00 (n=1511)
00/01 (n=1402)
01/02 (n=1546)
03 (n=1279)
04 (n=1290)
Study Year
Moxifloxacin
Levofloxacin
Gemifloxacin
Gatifloxacin
Ciprofloxacin

Throughout CROSS, 20% of isolates were collected from children (<16 years), 43% from adults (16 – 64 years), and 38% from elderly patients (>64 years). The majority of the ciprofloxacin-resistant *S. pneumoniae* isolates (62%) were isolated from elderly patients while 35% were from adults and 3% from children. The annual ciprofloxacin resistance rates in each age group are presented in figure 3.2. Statistically significant increases in ciprofloxacin resistance were observed between 1997/98 and 2004 in the adults (p=0.001) and the elderly patients (p=0.0001).

The isolates were collected from geographically diverse regions of Canada. The number of isolates received from each province was as follows with the number of centres located in each province indicated in parentheses: 12 from British Columbia (2 centres), 18 from Alberta (2 centres), 11 from Saskatchewan (2 centres), 27 from Manitoba (2 centres), 35 from Ontario (5 centres), 40 from Quebec (5 centres), 4 from Nova Scotia (1 centre), 8 from New Brunswick (2 centres), and 1 from Prince Edward Island (1 centre). The annual ciprofloxacin resistance rates in each province are presented in figure 3.3. The descending order of ciprofloxacin resistance by province during the course of this study was Manitoba (2.4%), Quebec (2.1%), New Brunswick (1.7%), Ontario (1.6%), Alberta (1.4%), British Columbia (1.3%), Saskatchewan (1.1%), Nova Scotia (0.9%), and Prince Edward Island (0.3%).

Figure 3.2 **Ciprofloxacin resistance of Canadian clinical *S. pneumoniae* isolates per year (1997/98 – 2004) by age group. Age groups were defined as: children (0-15 years), adults (16-64 years), and elderly (>64 years). Isolates were collected and MICs were tested by microbroth dilution as part of CROSS. The number of isolates collected each year is indicated in parentheses.**

Fig. 3.2

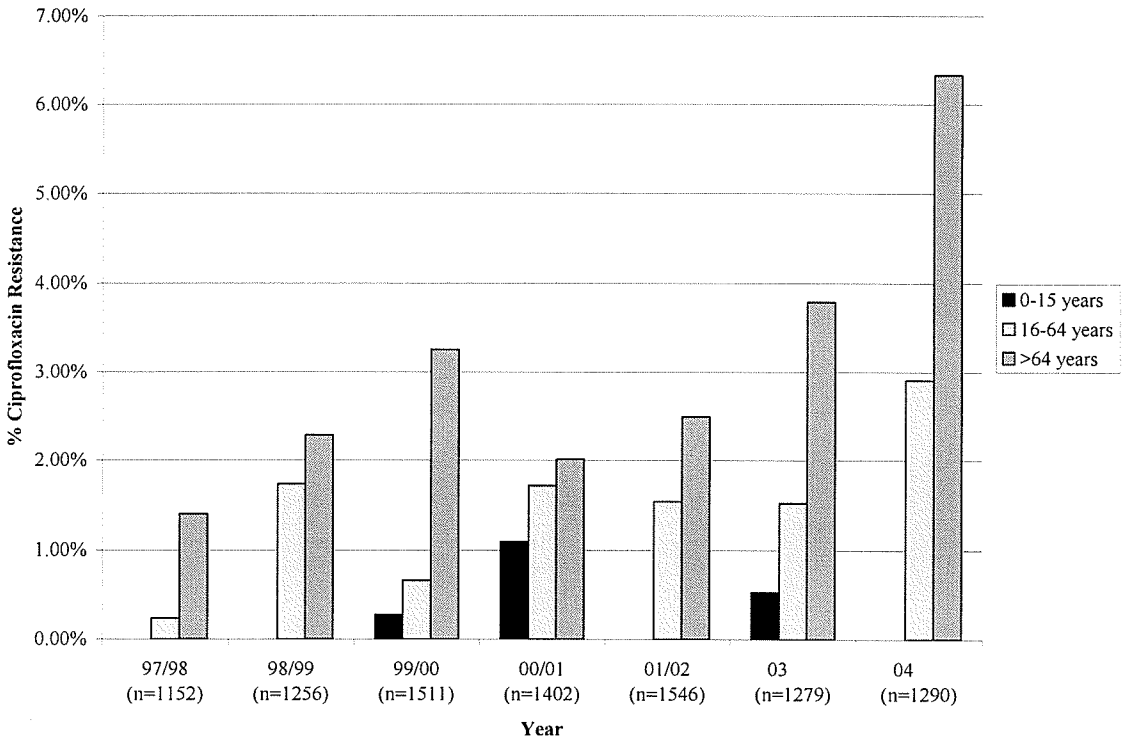
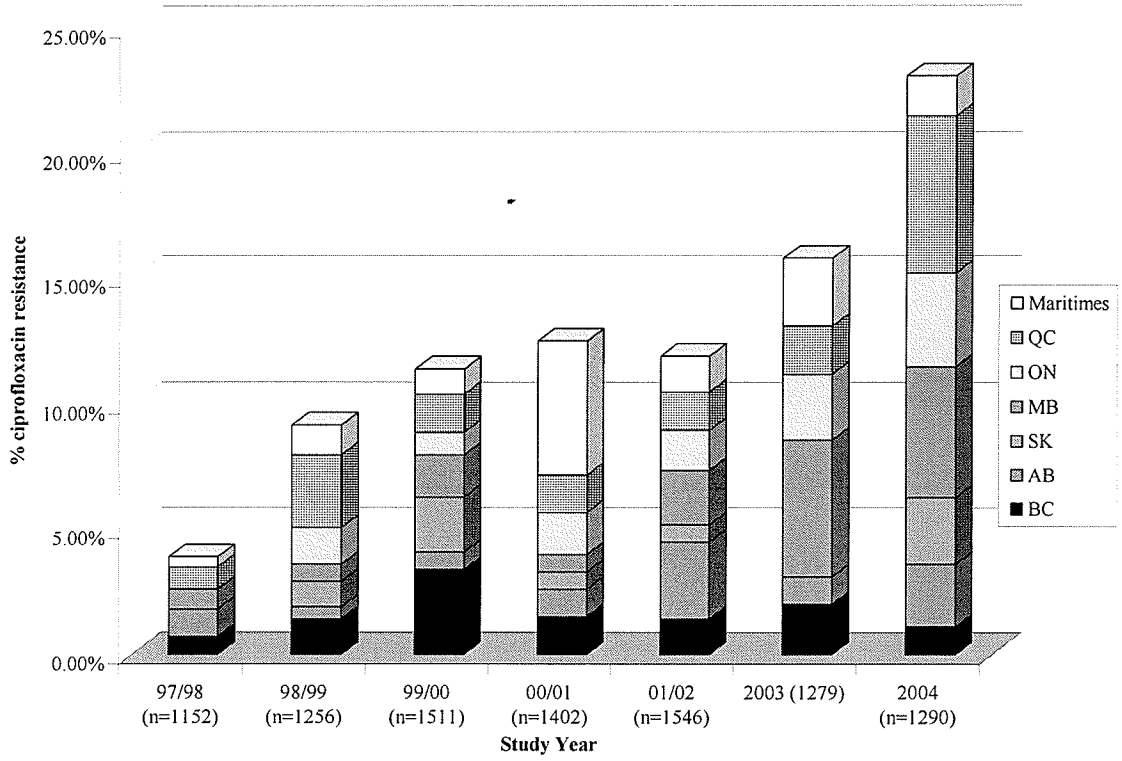

7.00%
6.00%
5.00%
4.00%
3.00%
2.00%
1.00%
0.00%
% Ciprofloxacin Resistance
0-15 years
16-64 years
>64 years
97/98 (n=1152)
98/99 (n=1256)
99/00 (n=1511)
00/01 (n=1402)
01/02 (n=1546)
03 (n=1279)
04 (n=1290)
Year

Figure 3.3 **Ciprofloxacin resistance in Canadian provinces by year (1997/98 to 2004). Isolates were collected and MICs were tested by microbroth dilution as part of CROSS. The number of isolates collected each year is indicated in parentheses. QC, Quebec; ON, Ontario; MB, Manitoba; SK, Saskatchewan; AB, Alberta; BC, British Columbia**

Fig. 3.3


25.00%
20.00%
15.00%
10.00%
5.00%
0.00%
% ciprofloxacin resistance
Maritimes
QC
ON
MB
SK
AB
BC
97/98 (n=1152)
98/99 (n=1256)
99/00 (n=1511)
00/01 (n=1402)
01/02 (n=1546)
2003 (1279)
2004 (n=1290)
Study Year

b. Antibiogram

The MICs of numerous antimicrobials were determined for the ciprofloxacin-resistant *S. pneumoniae* isolates. The MICs of ciprofloxacin, gatifloxacin, gemifloxacin, levofloxacin, moxifloxacin, penicillin, clarithromycin, and cefotaxime are included in appendix B. Of the 156 ciprofloxacin-resistant *S. pneumoniae*, 75 (48%), 8 (5%), 84 (54%), and 25 (16%) were also resistant to gatifloxacin, gemifloxacin, levofloxacin, and moxifloxacin, respectively. Among the ciprofloxacin-resistant isolates, 17 (11%) were resistant to penicillin, 41 (26%) were resistant to clarithromycin, 2 (1%) were resistant to cefotaxime, and 10 (6%) were resistant to both penicillin and clarithromycin.

c. Molecular Characterization

All 156 ciprofloxacin-resistant *S. pneumoniae* isolates were molecularly characterized. The isolates were serotyped, analyzed for GyrA and ParC QRDR substitutions and efflux, and they were fingerprinted using PFGE. The serotypes, QRDR substitutions and efflux results are presented in table 3.1. The molecular characterization of isolates collected between 1997/98 and 2000 has been published (137).

i. Sequencing of the Quinolone Resistance-Determining Regions of *gyrA* and *parC*

Of the 156 ciprofloxacin-resistant *S. pneumoniae* isolates, 16 (10%) had no substitutions in the QRDRs of either GyrA or ParC, 40 (26%) had a QRDR ParC substitution, 3 (2%) had a QRDR GyrA substitution, and 97 (62%) had substitutions in the QRDRs of both GyrA and ParC. The specific substitutions observed in ParC included: Ser79Ala, Ser79Phe, Ser79Tyr, Asp83Ala, Asp83Asn, Asp83Gly, and Asp83Tyr. The substitution observed in GyrA was Ser81Phe. The combinations of

Table 3.1 **Serotype, substitutions in the QRDRs of GyrA and ParC, and efflux of the 156 ciprofloxacin-resistant *S. pneumoniae* isolates collected across Canada between 1997/8 and 2004**

Isolate	Serotype	GyrA QRDR Substitutions	ParC QRDR Substitutions	Efflux[a]
1282	14	None observed	Lys137Asn	*4*
4030	6B	**Ser81Phe**	**Asp83Asn**	*4*
4610	33F	None observed	**Ser79Phe**	0
9989	23F	**Ser81Phe**	**Ser79Phe**	2
10277	11A	None observed	**Ser79Phe**	*4*
10280	6A	**Ser81Phe**	**Ser79Phe**	2
10733	6B	None observed	**Ser79Phe**	2
11361	11A	**Ser81Phe**	**Ser79Phe**	2
11434	23F	None observed	**Ser79Phe**, Lys137Asn	2
12291	11A	None observed	**Ser79Tyr**	2
12818	4	**Ser81Phe**	**Asp83Tyr**	*4*
12873	11A	None observed	**Ser79Phe**	*4*
12883	9N	None observed	**Ser79Phe**	2
14033	22F	**Ser81Phe**	**Ser79Tyr**	2
14744	22F	None observed	**Ser79Phe**	2
14769	14	None observed	**Ser79Phe**, Lys137Asn	2
14904	23F	**Glu85Lys**	**Ser79Phe**, Lys137Asn	2
15017	15B	None observed	None observed	*4*
16071	23A	**Ser81Phe**	**Ser79Tyr**	2
16072	19F	None observed	None observed	*4*
16078	6A	**Ser81Phe**	**Ser79Phe**	*4*
17012	19F	**Ser81Phe**	**Ser79Phe**	0
17913	23F	None observed	**Asp83Gly**	*4*
18397	6A	**Ser81Phe**	**Ser79Phe**	0
18410	19F	**Ser81Phe**	**Ser79Phe**	0
18955	11A	**Glu85Lys**	**Ser79Phe**	2
19103	10A	None observed	None observed	2
19120	19F	None observed	**Asp83Asn**	*4*
20336	9V	None observed	**Asp83Ala**, Lys137Asn	2
20709	6B	**Ser81Tyr**	**Ser79Phe**	2
21181	22F	**Glu85Lys**	**Ser79Phe**	2
21288	19F	**Glu85Lys**	**Ser79Phe, Asp83Tyr**	*4*
22350	14	**Ser81Phe**	**Ser79Phe**, Lys137Asn	0
22360	9V	None observed	**Asp83Ala**, Lys137Asn	2
22366	14	**Ser81Phe**	**Ser79Phe**, Lys137Asn	0
22627	17F	None observed	**Asp83Gly**	2
22668	11A	None observed	**Ser79Tyr**	2

22901	22F	**Ser81Phe**	**Asp83Asn**, Glu120Gln	2
23070	9N	**Ser81Phe**	**Ser79Phe**, Lys137Asn	*4*
23335	22F	**Ser81Phe**	**Ser79Phe**	2
23448	14	None observed	**Ser79Phe**	2
23574	12F	**Ser81Phe**	**Ser79Phe**, Lys137Asn	0
23786	22F	**Ser81Phe**	None observed	2
24086	9V	None observed	Tyr59Asp	2
25074	19F	None observed	**Ser79Phe**	2
25268	18C	**Ser81Phe**	None observed	2
26608	2	**Ser81Phe**	**Ser79Phe**	2
27224	6A	**Ser81Tyr**	**Ser79Phe**	*4*
27396	16F	**Ser81Phe**	**Ser79Phe**	*4*
27546	22F	**Ser81Phe**	**Ser79Tyr**	2
27833	19A	**Glu85Lys**	**Ser79Tyr**	2
27908	9V	None observed	**Asp83Asn**, Lys137Asn	2
27917	9V	None observed	**Asp83Asn**, Lys137Asn	2
28374	19A	**Glu85Lys**	**Ser79Tyr**	2
28669	6B	None observed	Ser107Tyr	2
29012	19F	None observed	None observed	*4*
29111	19F	**Ser81Phe**	**Ser79Phe**, Lys137Asn	*4*
29228	19F	**Ser81Phe**	**Asp83Tyr**	2
29262	NT	None observed	Lys137Asn	*4*
29265	4	**Glu85Lys**	**Ser79Phe**	*4*
29516	22F	**Ser81Phe**	**Ser79Tyr, Asp83Asn**	0
29927	6B	**Ser81Phe**	**Ser79Phe**	2
30890	6A	None observed	None observed	*4*
30900	6B	**Ser81Phe**	**Ser79Phe**	0
31685	9V	**Ser81Phe**	**Ser79Phe**, Lys137Asn	2
32534	18B	**Ser81Tyr**	**Ser79Tyr**	0
32549	19F	None observed	**Asp83Asn**	2
32839	6B	**Ser81Phe**	**Ser79Tyr**	2
32867	23F	**Ser81Phe**	**Ser79Phe**	0
33035	11D	**Ser81Phe**	**Asp83Asn**	2
33726	9N	**Ser81Phe**	**Ser79Phe**, Lys137Asn	2
33809	6B	**Ser81Phe**	**Ser79Tyr**	2
34547	15C	**Ser81Phe**	**Ser79Tyr**	2
34549	15C	**Ser81Phe**	**Ser79Phe**	2
34572	19F	**Ser81Phe**	**Ser79Phe**	0
34604	22F	None observed	**Ser79Tyr**	2
35097	6B	**Ser81Tyr**	**Ser79Phe**	0
35152	12F	None observed	**Ser79Phe**, Lys137Asn	2
39710	19F	None observed	None observed	*8*
40810	4	**Ser81Phe**	**Ser79Phe**	0

42372	23F	**Glu85Gly**	**Ser79Phe**	2
44171	11A	None observed	None observed	*4*
45089	31	None observed	Lys137Asn	*4*
45333	23F	**Ser81Phe**	**Ser79Phe**, Lys137Asn	2
45336	18C	**Ser81Phe**	**Ser79Phe**	0
45693	3	**Ser81Phe**	**Ser79Phe**	*4*
45776	10A	**Ser81Phe**	**Ser79Phe**	2
45780	6A	None observed	**Ser79Tyr**	2
46970	22F	**Ser81Phe**	**Asp83Asn**	2
47209	19F	None observed	**Ser79Phe**	2
47224	19F	None observed	**Ser79Phe**	2
47225	19F	None observed	**Ser79Phe**	2
47396	9V	**Ser81Phe**	**Ser79Phe**, Lys137Asn	0
47789	19F	None observed	**Ser79Phe**	2
47797	22F	**Glu85Lys**	**Ser79Phe, Asp83Tyr**	2
48198	12F	**Ser81Phe**	**Ser79Phe**, Lys137Asn	2
48486	22F	**Ser81Phe**	**Ser79Tyr**	2
48865	12F	**Ser81Phe**	**Ser79Phe**, Lys137Asn	2
48866	12F	**Ser81Phe**	**Ser79Phe**, Lys137Asn	2
49101	19F	**Ser81Phe**	**Ser79Phe**, Lys137Asn	2
49322	19A	**Ser81Phe**	**Ser79Phe**	2
49710	3	**Ser81Phe**	**Ser79Phe**	*4*
49711	3	**Ser81Phe**	**Ser79Phe**	*4*
49755	12F	**Ser81Phe**	**Ser79Phe**, Lys137Asn	0
49773	12F	**Ser81Phe**	**Ser79Phe**, Lys137Asn	2
49928	12F	**Ser81Phe**	**Ser79Phe**, Lys137Asn	2
50154	15C	None observed	**Ser79Phe**	*4*
50227	15B	**Glu85Lys**	**Asp83Asn**	0
50418	23F	**Ser81Phe**	**Ser79Phe**	2
50770	22F	**Ser81Tyr**	**Ser79Tyr**	2
50835	6B	**Ser81Phe**	**Ser79Phe**	2
50946	19F	**Ser81Phe**	**Ser79Phe**	2
51126	19A	**Ser81Phe**	**Ser79Phe**	0
51531	11A	**Ser81Phe**	None observed	*4*
51597	23A	None observed	**Ser79Phe**	*4*
52418	19A	**Ser81Phe**	**Ser79Tyr**, Lys137Asn	2
52651	35B	**Ser81Phe**	**Ser79Phe, Asp83Asn**	2
52941	6A	**Ser81Phe**	**Ser79Phe**	2
53155	12F	**Ser81Phe**	**Ser79Phe**, Lys137Asn	2
53482	9A	**Ser81Phe**	**Ser79Phe**, Lys137Asn	2
53683	9N	**Ser81Phe**	**Ser79Tyr**, Lys137Asn	*4*
53908	6B	**Ser81Phe**	**Ser79Tyr**	0
54610	12F	**Ser81Phe**	**Ser79Phe**, Lys137Asn	2

54883	33F	**Ser81Phe**	**Ser79Phe**	0
55072	12F	None observed	**Ser79Phe**, Lys137Asn	2
55073	11A	None observed	None observed	*4*
55160	6B	**Ser81Phe**	**Ser79Phe**	0
55175	6A	**Ser81Phe**	**Ser79Phe**	0
55178	6A	**Ser81Phe**	**Ser79Phe**	0
55335	34	None observed	None observed	*4*
55361	6B	None observed	**Ser79Tyr**	2
55374	14	**Glu85Lys**	**Ser79Tyr**, Lys137Asn	2
55430	11A	**Ser81Tyr**	**Ser79Phe**, Lys137Asn	2
55431	12F	**Ser81Tyr**	**Ser79Phe**, Lys137Asn	0
55660	23F	**Ser81Phe**	**Ser79Phe**	0
55663	23F	**Ser81Phe, Glu85Lys**	**Ser79Phe**, Lys137Asn	2
55666	23F	**Glu85Lys**	**Ser79Phe**	2
55719	6A	None observed	**Ser79Ala**	2
55798	6A	None observed	None observed	*8*
56276	10A	**Glu85Lys**	**Ser79Phe**	*4*
56283	19A	**Ser81Phe**	**Ser79Phe**	2
56298	11A	**Ser81Phe**	Asp78Ala	2
56301	19F	None observed	**Asp83Tyr**	2
56304	19F	None observed	**Asp83Tyr**	2
56319	22F	None observed	**Ser79Phe**	2
56336	19F	None observed	**Ser79Phe**	2
56414	23F	None observed	**Asp83Asn**	2
56419	10A	**Glu85Lys**	**Ser79Phe**	2
56604	10A	**Ser81Phe**	**Ser79Phe**	0
56745	9N	None observed	Lys137Asn	2
56765	20	None observed	**Asp83Asn**	0
56782	20	**Ser81Phe**	**Ser79Tyr**	*4*
56904	23F	**Ser81Phe**	**Ser79Phe**, Lys137Asn	2
57155	22F	None observed	**Ser79Tyr**	2
57272	14	**Glu85Lys**	**Ser79Phe**	2
57278	9V	None observed	**Asp83Asn**, Lys137Asn	2

[a], fold decrease in MIC in the presence of an efflux inhibitor; Bold, QRDR substitutions associated with resistance; Italics, Efflux-positive; NT, non-typeable

substitutions observed in ParC and GyrA included: Ser79Phe and Ser81Phe, Ser79Phe and Ser81Tyr, Ser79Phe and Glu85Lys, Ser79Phe and Glu85Gly, Ser79Tyr and Ser81Phe, Ser79Tyr and Ser81Tyr, Ser79Tyr and Glu85Lys, Asp83Asn and Ser81Phe, Asp83Asn and Glu85Lys, Asp83Tyr and Ser81Phe, and Asp78Ala and Ser81Phe. A small number of isolates had 3 substitutions in ParC and GyrA, including Ser79Phe/Asp83Tyr and Glu85Lys, Ser79Phe/Asp83Asn and Ser81Phe, Ser79Tyr/Asp83Asn and Ser81Phe, and Ser79Phe and Ser81Phe/Glu85Lys. The percent of isolates with each of the aforementioned substitutions is presented in figure 3.4a. Figure 3.4b displays the percent of isolates with combined substitutions at each altered amino acid. For example, ParC substitutions Ser79Phe and Ser79Tyr are combined as Ser79.

The most common single ParC substitutions occurred at position Ser79 (17% of all ciprofloxacin-resistant isolates) with Ser79Phe observed most frequently (13% of all ciprofloxacin-resistant isolates). All isolates with single GyrA substitutions (2%) displayed the Ser81Phe alteration. The most commonly observed genotype had ParC and GyrA substitutions at positions Ser79 and Ser81 (46% of all ciprofloxacin-resistant isolates) with Ser79Phe and Ser81Phe as the most frequently observed combination (35% of all ciprofloxacin-resistant isolates). Isolates with 3 QRDR substitutions in GyrA and ParC were rare (3% of all ciprofloxacin-resistant isolates).

Figure 3.4 **Percent of the 156 ciprofloxacin-resistant *S. pneumoniae* isolates with resistance-associated QRDR substitutions in GyrA and/or ParC presented by (a) specific substitutions and (b) summary of substitutions at each altered amino acid. The number of isolates with each substitution is indicated in parentheses. Black bars indicate ParC substitutions. Light grey bars indicate GyrA substitutions. Grey bars indicate substitutions in GyrA and ParC.**

Fig. 3.4a

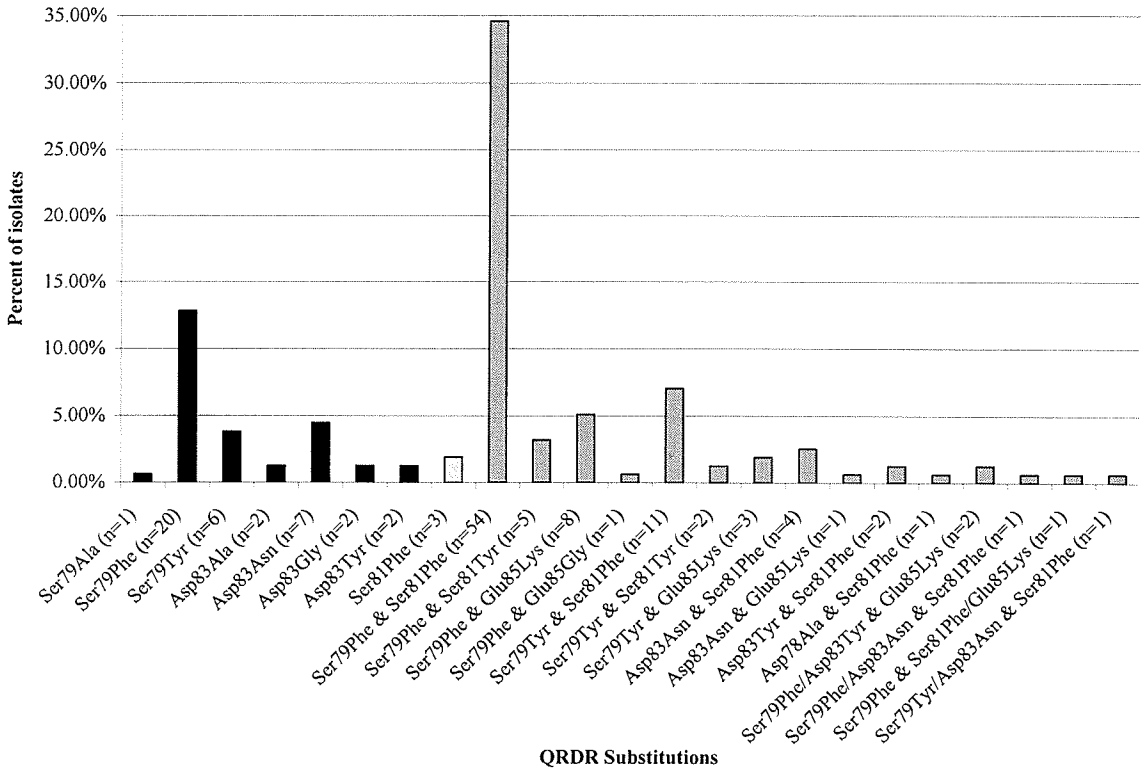
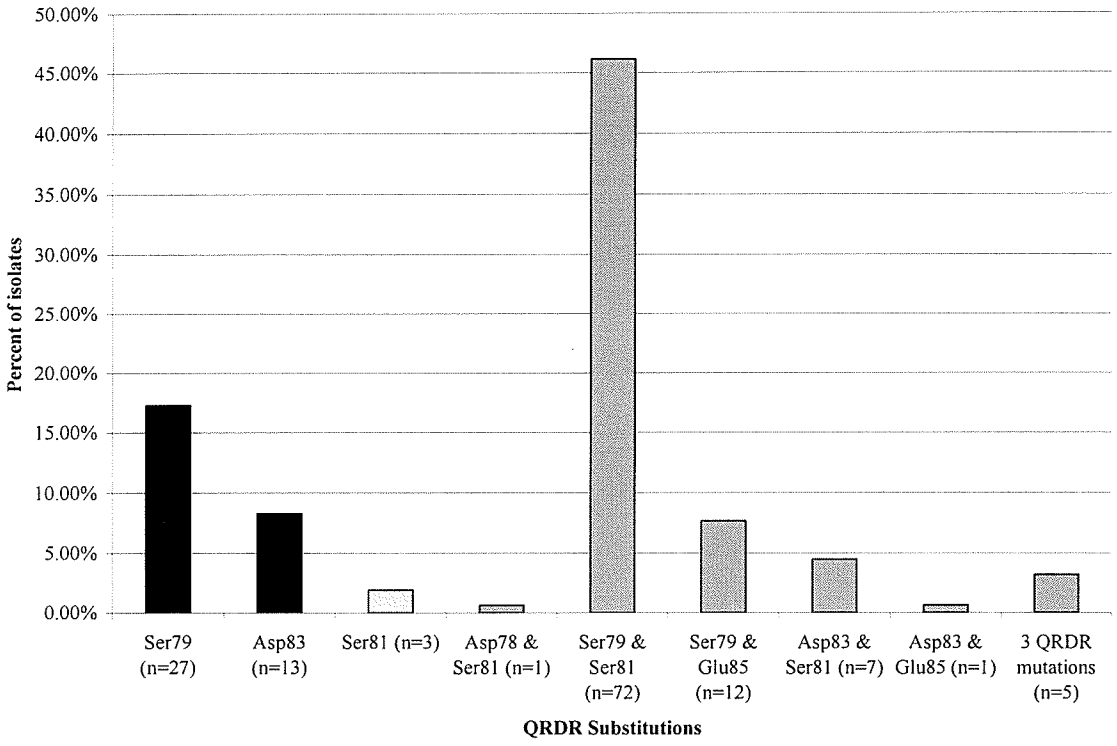

35.00%
30.00%
25.00%
20.00%
15.00%
10.00%
5.00%
0.00%
Percent of isolates
Ser79Ala (n=1)
Ser79Phe (n=20)
Ser79Tyr (n=6)
Asp83Ala (n=2)
Asp83Asn (n=7)
Asp83Gly (n=2)
Asp83Tyr (n=2)
Ser81Phe (n=3)
Ser79Phe & Ser81Phe (n=54)
Ser79Phe & Ser81Tyr (n=5)
Ser79Phe & Glu85Lys (n=8)
Ser79Phe & Glu85Gly (n=1)
Ser79Tyr & Ser81Phe (n=11)
Ser79Tyr & Ser81Tyr (n=2)
Ser79Tyr & Glu85Lys (n=3)
Asp83Asn & Ser81Phe (n=4)
Asp83Asn & Glu85Lys (n=1)
Asp83Tyr & Ser81Phe (n=2)
Asp78Ala & Ser81Phe (n=1)
Ser79Phe/Asp83Tyr & Glu85Lys (n=2)
Ser79Phe/Asp83Asn & Ser81Phe (n=1)
Ser79Phe & Ser81Phe/Glu85Lys (n=1)
Ser79Tyr/Asp83Asn & Ser81Phe (n=1)
QRDR Substitutions

Fig. 3.4b


50.00%
45.00%
40.00%
35.00%
30.00%
25.00%
20.00%
15.00%
10.00%
5.00%
0.00%
Percent of isolates
Ser79 (n=27)
Asp83 (n=13)
Ser81 (n=3)
Asp78 & Ser81 (n=1)
Ser79 & Ser81 (n=72)
Ser79 & Glu85 (n=12)
Asp83 & Ser81 (n=7)
Asp83 & Glu85 (n=1)
3 QRDR mutations (n=5)
QRDR Substitutions

In order to assess changes overtime, the percent of ciprofloxacin-resistant isolates with no QRDR substitutions, a single ParC substitution, a single GyrA substitution or substitutions in both GyrA and ParC are presented by year between 1997/98 and 2004 in figure 3.5. The percent of ciprofloxacin-resistant isolates without substitutions in the QRDRs of GyrA or ParC by year (1997/98 – 2004) were 25%, 7%, 9%, 19%, 14%, 3%, and 9%. The percent of isolates with substitutions in ParC each year was 25%, 57%, 27%, 19%, 14%, 19%, and 28%. The isolates with GyrA substitutions were observed in 2000/01 and 2004. The percent of isolates with GyrA and ParC substitutions each year was 50%, 36%, 63%, 52%, 71%, and 60%.

The percent of ciprofloxacin-resistant isolates with no QRDR substitutions, a single ParC substitution, a single GyrA substitution or substitutions in both GyrA and ParC were analyzed based on fluoroquinolone MICs. The percent of ciprofloxacin-resistant isolates with QRDR substitutions is presented based on ciprofloxacin, gatifloxacin, gemifloxacin, levofloxacin, and moxifloxacin MICs in figures 3.6 a, b, c, d, and e, respectively. The susceptible, intermediate, and resistant breakpoints are indicated on the gatifloxacin, gemifloxacin, levofloxacin, and moxifloxacin figures. The inclusion of the breakpoints highlights the concern that many isolates considered to be fluoroquinolone-susceptible carry resistance-associated mutations. These isolates could become highly fluoroquinolone-resistant upon subsequent treatment with a fluoroquinolone. This concern was first noted due to these figures and led to the ensuing investigation of fluoroquinolone resistance-associated substitutions in fluoroquinolone susceptible isolates detailed in part II.

Figure 3.5 **Percent of 156 ciprofloxacin-resistant *S. pneumoniae* isolates with QRDR substitutions in GyrA and/or ParC over seven years (1997/8 to 2004). The number of ciprofloxacin-resistant isolates collected each year is indicated in parentheses.**

Fig. 3.5

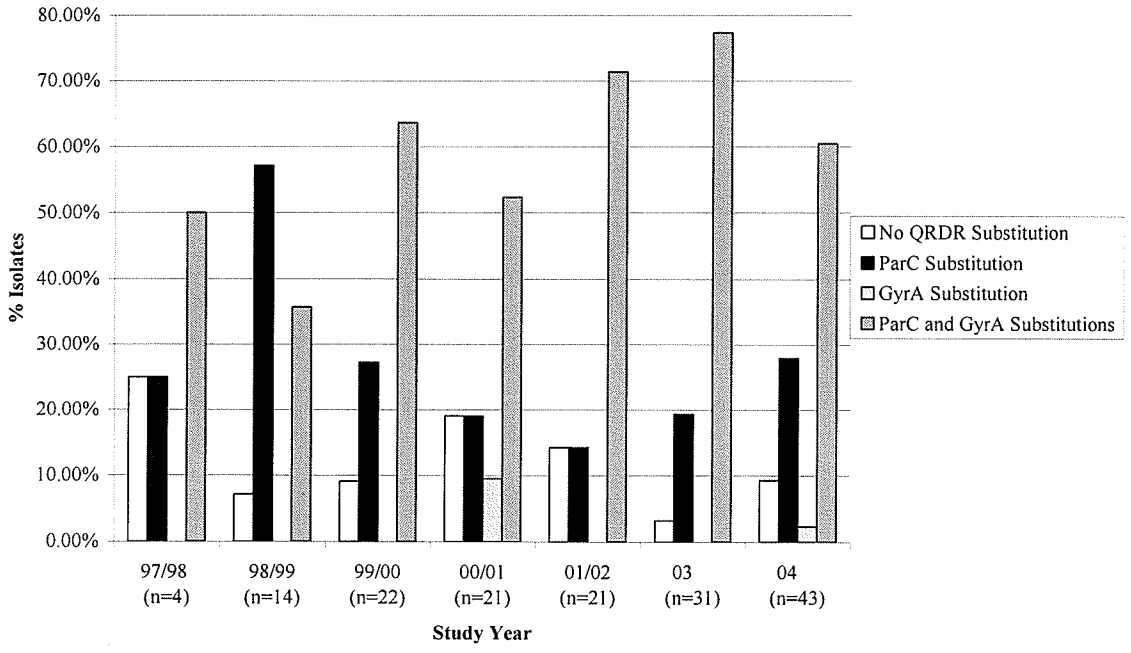

80.00%
70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
% Isolates
97/98 (n=4)
98/99 (n=14)
99/00 (n=22)
00/01 (n=21)
01/02 (n=21)
03 (n=31)
04 (n=43)
Study Year
No QRDR Substitution
ParC Substitution
GyrA Substitution
ParC and GyrA Substitutions

Figure 3.6 **Percent of the 156 ciprofloxacin-resistant *S. pneumoniae* isolates with QRDR substitutions by (a) ciprofloxacin (b) gatifloxacin (c) gemifloxacin (d) levofloxacin, and (e) moxifloxacin MICs. The number of isolates at each MIC is indicated in parentheses. The susceptible (S), intermediate (I), and resistant (R) categories are indicated on the graphs except for the ciprofloxacin graph as all isolates are ciprofloxacin-resistant.**

Fig. 3.6a

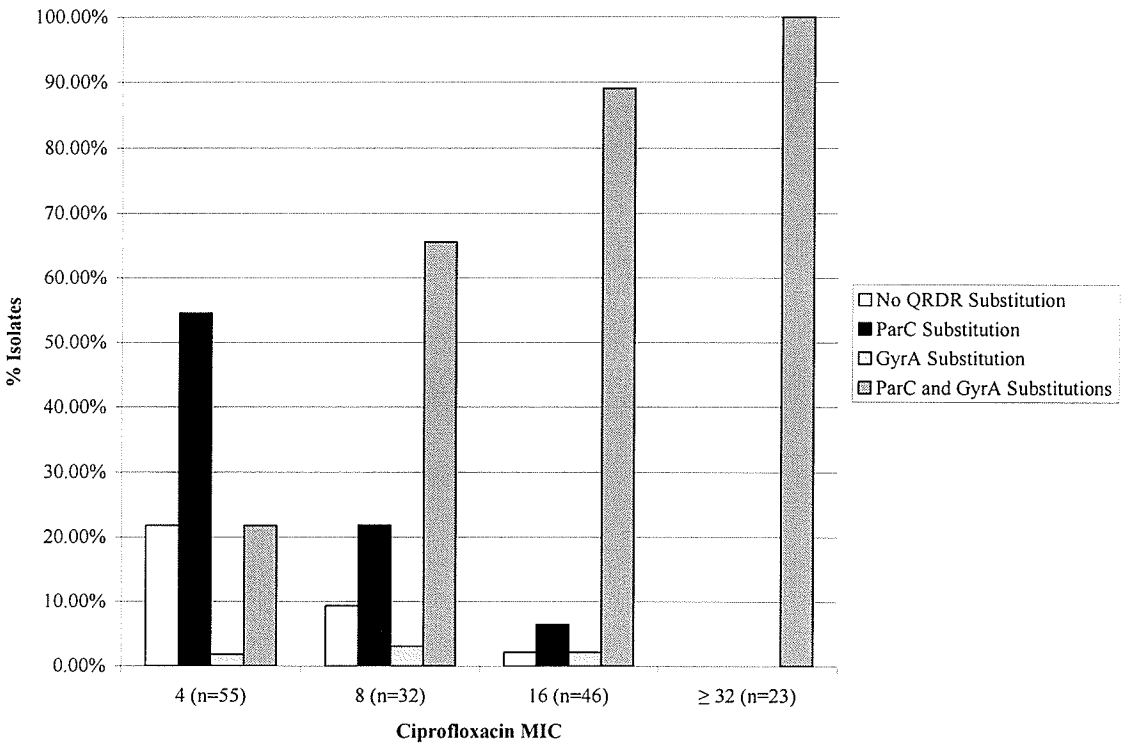

100.00%
90.00%
80.00%
70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
% Isolates
4 (n=55)
8 (n=32)
16 (n=46)
≥ 32 (n=23)
Ciprofloxacin MIC
No QRDR Substitution
ParC Substitution
GyrA Substitution
ParC and GyrA Substitutions

Fig. 3.6b

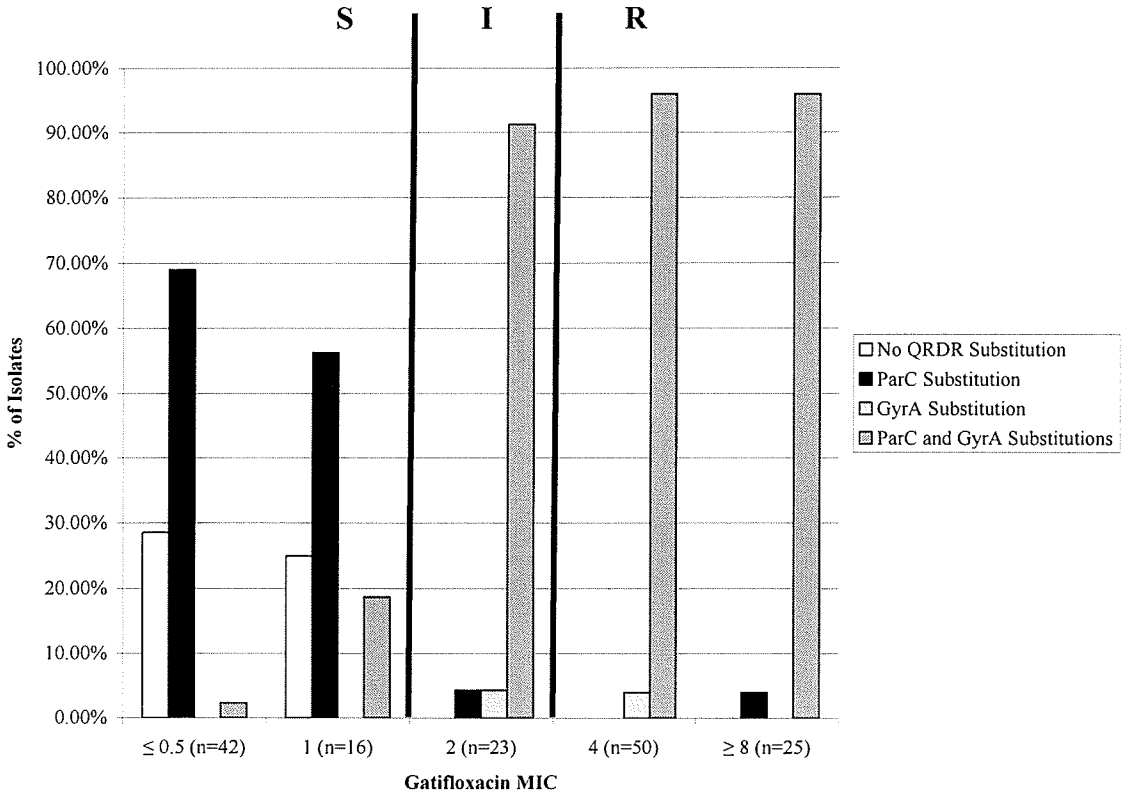

S
I
R
100.00%
90.00%
80.00%
70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
% of Isolates
≤ 0.5 (n=42)
1 (n=16)
2 (n=23)
4 (n=50)
≥ 8 (n=25)
Gatifloxacin MIC
No QRDR Substitution
ParC Substitution
GyrA Substitution
ParC and GyrA Substitutions

Fig. 3.6c

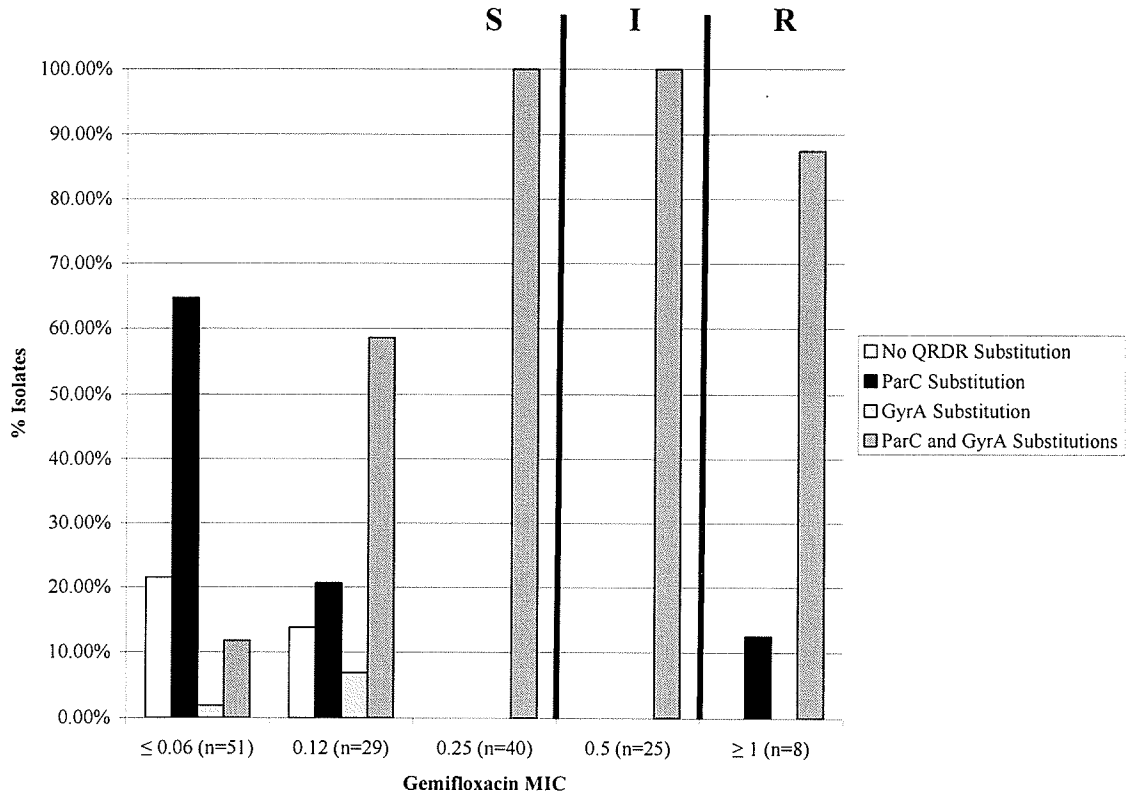

S
I
R
100.00%
90.00%
80.00%
70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
% Isolates
≤ 0.06 (n=51)
0.12 (n=29)
0.25 (n=40)
0.5 (n=25)
≥ 1 (n=8)
Gemifloxacin MIC
No QRDR Substitution
ParC Substitution
GyrA Substitution
ParC and GyrA Substitutions

Fig. 3.6d

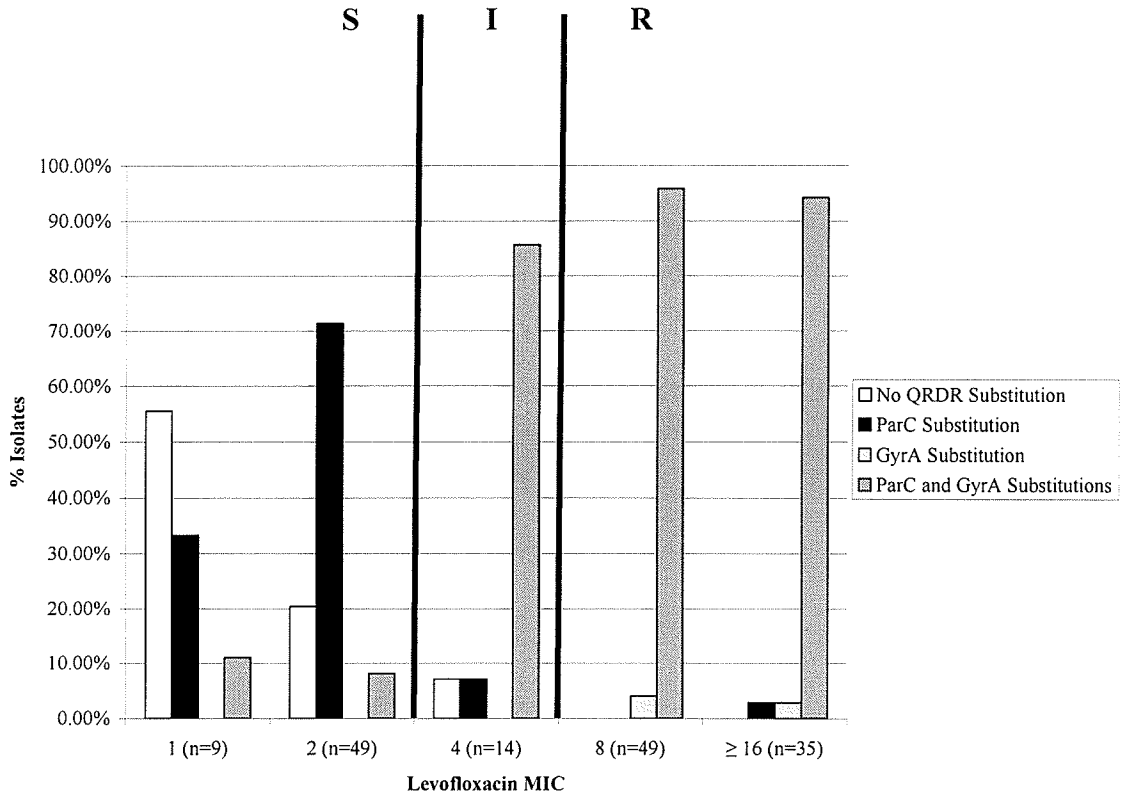
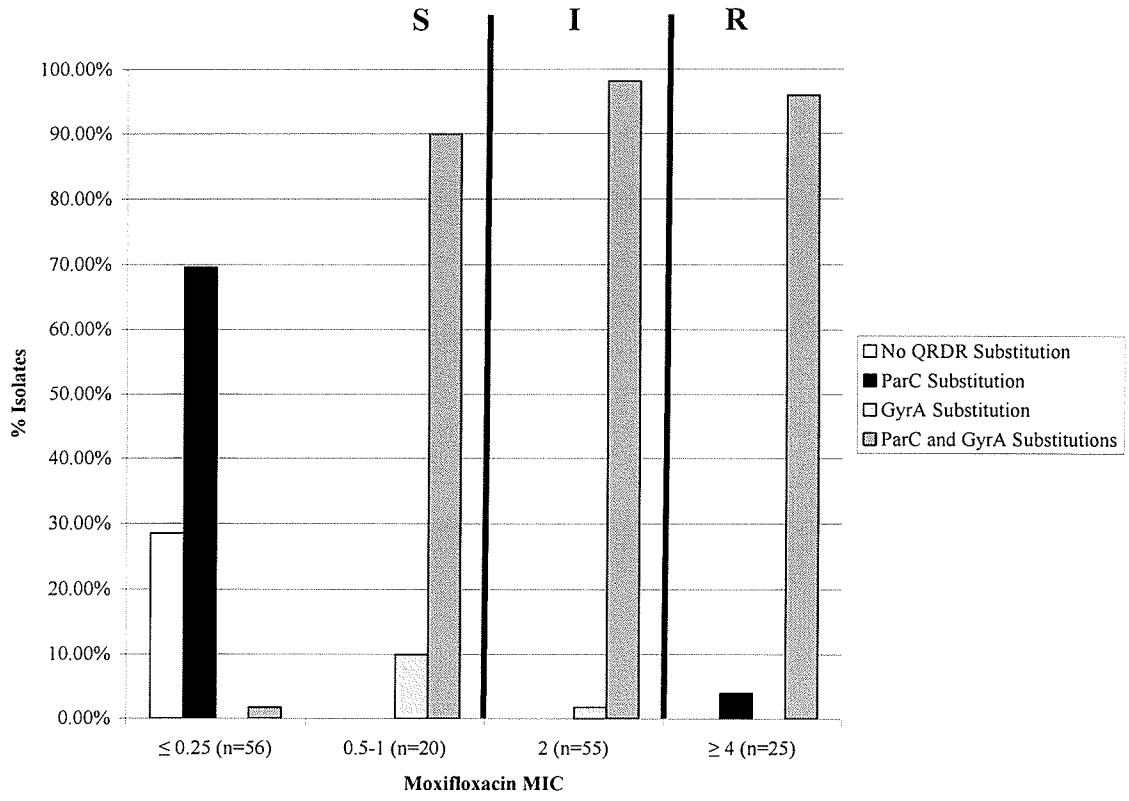

S
I
R
100.00%
90.00%
80.00%
70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
% Isolates
1 (n=9)
2 (n=49)
4 (n=14)
8 (n=49)
≥ 16 (n=35)
Levofloxacin MIC
No QRDR Substitution
ParC Substitution
GyrA Substitution
ParC and GyrA Substitutions

Fig. 3.6e


S
I
R
100.00%
90.00%
80.00%
70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
% Isolates
≤ 0.25 (n=56)
0.5-1 (n=20)
2 (n=55)
≥ 4 (n=25)
Moxifloxacin MIC
No QRDR Substitution
ParC Substitution
GyrA Substitution
ParC and GyrA Substitutions

At ciprofloxacin MICs of 4, 8, 16, and 32 μg/mL, 22%, 9%, 2%, and 0% of isolates had no QRDR substitutions in GyrA and ParC. Fifty-five percent, 9%, 2%, and 0% of isolates at ciprofloxacin MICs of 4, 8, 16, and 32 μg/mL, respectively had single ParC substitutions. Conversely, 22%, 66%, 89%, and 100% of isolates had substitutions in GyrA and ParC at ciprofloxacin MICs of 4, 8, 16, and 32 μg/mL, respectively.

Only 29% and 25% of the ciprofloxacin-resistant isolates had no QRDR substitutions at gatifloxacin-susceptible MICs of ≤ 0.5 and 1 μg/mL, respectively. At these MICs, 69% and 56% of isolates had single ParC substitutions and 2% and 19% had substitutions in both GyrA and ParC. At gatifloxacin-intermediate and resistant MICs of ≥ 2 μg/mL, over 90% of the isolates had substitutions in GyrA and ParC. Isolates with single GyrA substitutions were gatifloxacin-intermediate or resistant.

The isolates with single GyrA substitutions had gemifloxacin-susceptible MICs of 0.06 and 0.12 μg/mL. Within the gemifloxacin-susceptible MICs, the percent of isolates without QRDR substitutions ranged from 22% (MIC ≤ 0.06 μg/mL) to 0% (MIC 0.25 μg/mL). The percent of isolates with single ParC substitutions ranged from 65% to 0% and the percent of isolates with substitutions in GyrA and ParC ranged from 12% to 100% between the MICs of ≤ 0.06 and 0.25 μg/mL. Over 87% of gemifloxacin-intermediate and resistant isolates had substitutions in GyrA and ParC.

At levofloxacin-susceptible MICs of 1 and 2 μg/mL, 56% and 20% of isolates had no QRDR substitutions, 33% and 71% had single substitutions in ParC, and 11% and 8% had substitutions in GyrA and ParC, respectively. Over 86% of levofloxacin-intermediate isolates had substitutions in GyrA and ParC. Similarly, over 94% of

levofloxacin-resistant isolates had substitutions in GyrA and ParC. The isolates with GyrA substitutions were levofloxacin-resistant.

Isolates with GyrA substitutions were either moxifloxacin-susceptible or intermediate. In figure 3.6e, the isolates with moxifloxacin MICs of 0.5 and 1 μg/mL were combined as both MICs are considered susceptible and only 2 isolates in the study had a moxifloxacin MIC of 0.5 μg/mL. The percent of moxifloxacin-susceptible isolates with no QRDR substitutions ranged from 29% to 0% (MICs ≤ 0.25 – 1 μg/mL). The percent of moxifloxacin-susceptible isolates with ParC substitutions and GyrA and ParC substitutions ranged from 70% to 0% and 2% to 90% (MICs ≤ 0.25 – 1 μg/mL), respectively. Over 96% of moxifloxacin-intermediate and resistant isolates had substitutions in GyrA and ParC.

ii. Presence of Reserpine-Inhibited Efflux

The percent of efflux-positive ciprofloxacin-resistant *S. pneumoniae* isolates collected each year of CROSS, as depicted in figure 3.7a, decreased from 50% in 1997/98 to 19% in 2004 (p=0.19).

The percent of efflux-positive ciprofloxacin-resistant *S. pneumoniae* isolates are presented by ciprofloxacin MIC in figure 3.7b. The percent of efflux-positive isolates at ciprofloxacin MICs of 4, 8, 16, and 32 μg/mL were 27%, 31%, 13%, and 13%, respectively.

Figure 3.7c presents the percent of efflux-positive ciprofloxacin-resistant *S. pneumoniae* by QRDR genotype. Seventy-five percent of isolates with no QRDR substitutions were efflux-positive. Thirty-three percent of isolates with a substitution in

Figure 3.7 **Percent of efflux-positive ciprofloxacin-resistant *S. pneumoniae* isolates (a) per year between 1997/8 and 2004, (b) by ciprofloxacin MIC, and (c) by QRDR substitution. Efflux was tested by the reserpine-inhibition method. The number of isolates in each category: year, MIC or QRDR substitution, are indicated in parentheses.**

Fig. 3.7a

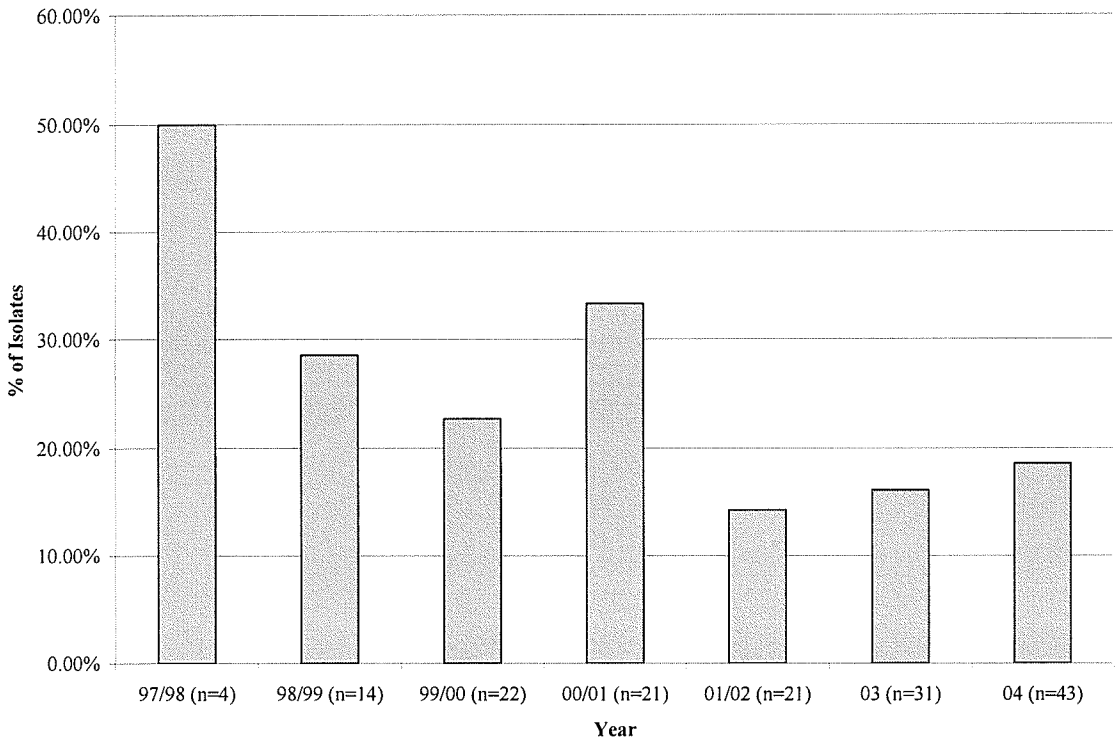

60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
% of Isolates
97/98 (n=4)
98/99 (n=14)
99/00 (n=22)
00/01 (n=21)
01/02 (n=21)
03 (n=31)
04 (n=43)
Year

Fig. 3.7b

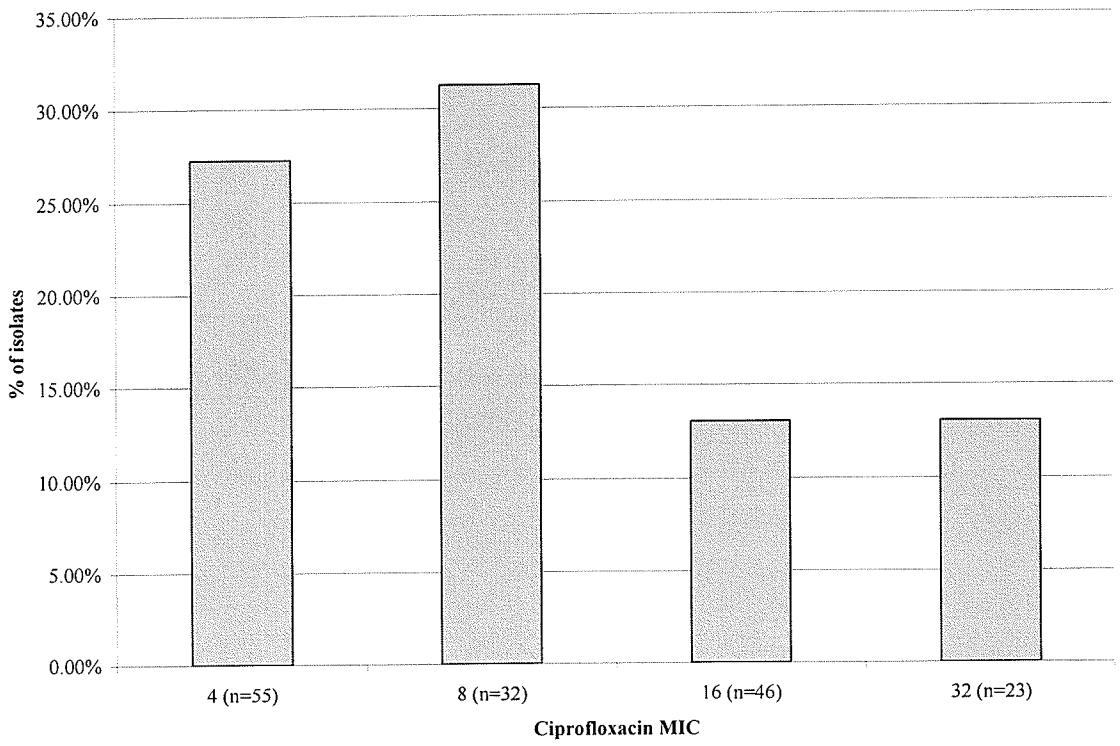
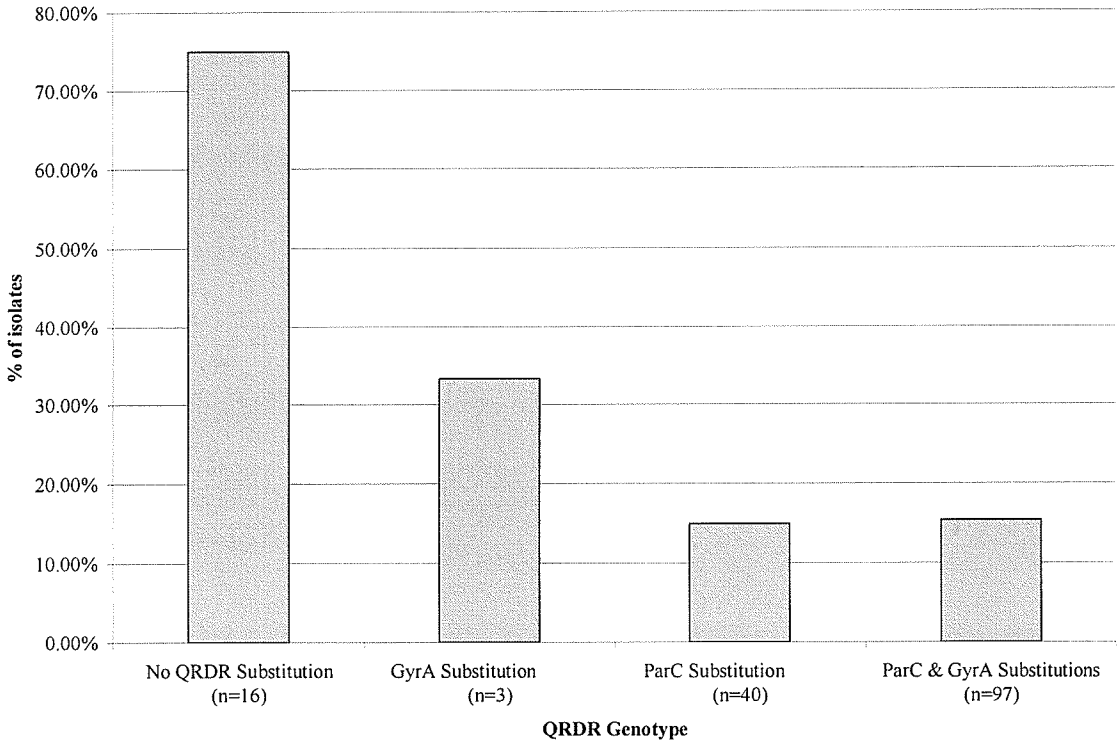

35.00%
30.00%
25.00%
20.00%
15.00%
10.00%
5.00%
0.00%
% of isolates
4 (n=55)
8 (n=32)
16 (n=46)
32 (n=23)
Ciprofloxacin MIC

Fig. 3.7c


80.00%
70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
% of isolates
No QRDR Substitution (n=16)
GyrA Substitution (n=3)
ParC Substitution (n=40)
ParC & GyrA Substitutions (n=97)
QRDR Genotype

GyrA were also efflux-positive. Fifteen percent of isolates with either single ParC substitutions or substitutions in GyrA and ParC were also efflux-positive.

iii. Serotype Analysis

The serotypes observed in the ciprofloxacin-resistant *S. pneumoniae* isolates were: 2, 3, 4, 6A, 6B, 9A, 9N, 9V, 10A, 11A, 11D, 12F, 14, 15B, 15C, 16F, 17F, 18B, 18C, 19A, 19F, 20, 22F, 23A, 23F, 31, 33F, 34, 35B, and non-typeable. The number of isolates with each serotype collected per year are shown in figure 3.8a.

Some serotypes occurred very infrequently and were only reported in one year. These serotypes and the year they were isolated, in parentheses, are: 2 (2000/01), 3 (2003), 9A (2004), 11D (1998-99), 16F (2000/01), 17F (1999/2000), 18B (1998/99), 20 (2004), 31 (2003), 34 (2004), 35B (2004), and non-typeable (2000/01).

The 10 most common serotypes observed were 6A, 6B, 9V, 11A, 12F, 14, 19A, 19F, 22F, and 23F. These serotypes represented 70 to 85% of the ciprofloxacin-resistant isolates collected every year. The number of isolates with these serotypes collected each year is shown in figure 3.8b. Yearly fluctuations with occasional dramatic increases and decreases in the prevalence of serotypes were observed. For example, for all years of study, serotype 6B represented 3 to 7.5% of the isolates except in 2001/02 when 24% of the isolates were 6B.

The serotypes of the ciprofloxacin-resistant isolates are presented in relation to the presence or absence of substitutions in the QRDRs of GyrA and/or ParC in figure 3.9. Certain serotypes were associated with one QRDR genotype. Serotypes 2, 3, 4, 9A, 11D, 16F, 18B, 19A, and 35B were only observed in isolates with QRDR substitutions in

Figure 3.8 **Serotypes of 156 ciprofloxacin-resistant *S. pneumoniae* isolates collected in Canada between 1997/8 and 2004 presented as (a) all serotypes and (b) the 10 most common serotypes. The number of isolates collected each year is indicated in parentheses. NT, non-typeable**

Fig. 3.8a

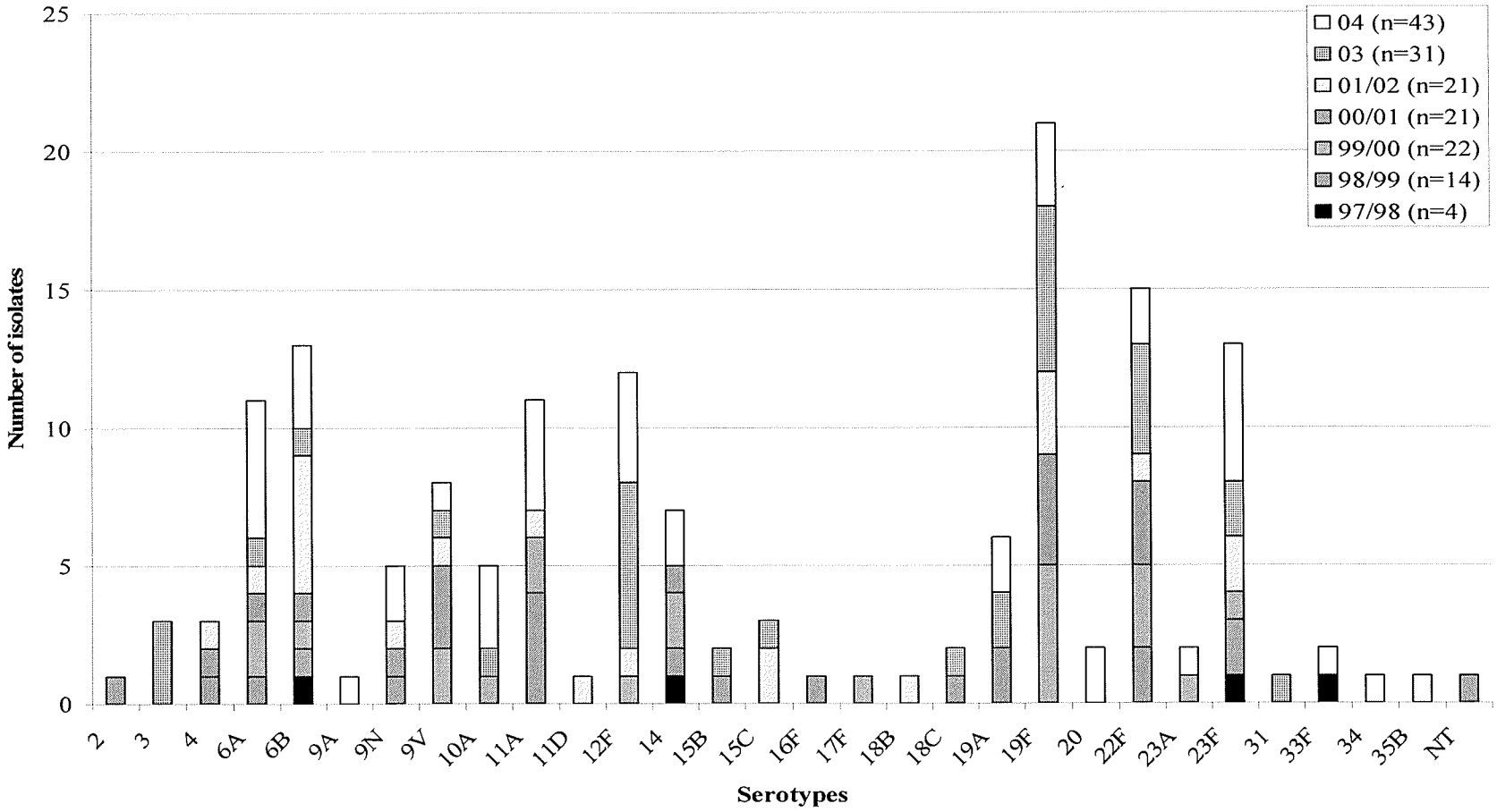

04 (n=43)
03 (n=31)
01/02 (n=21)
00/01 (n=21)
99/00 (n=22)
98/99 (n=14)
97/98 (n=4)
Number of isolates
0
5
10
15
20
25
2
3
4
6A
6B
9A
9N
9V
10A
11A
11D
12F
14
15B
15C
16F
17F
18B
18C
19A
19F
20
22F
23A
23F
31
33F
34
35B
NT
Serotypes

Fig. 3.8b

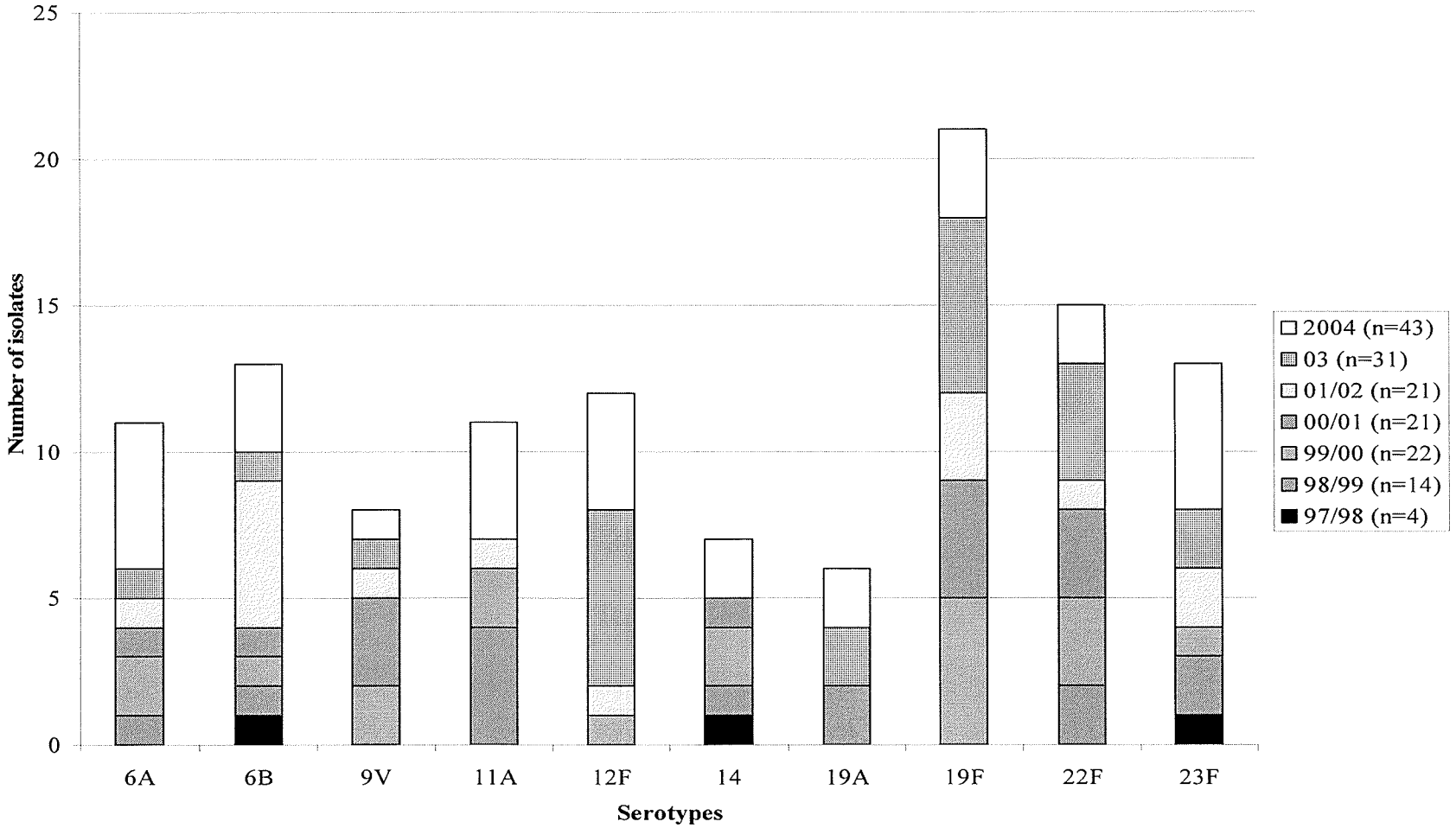
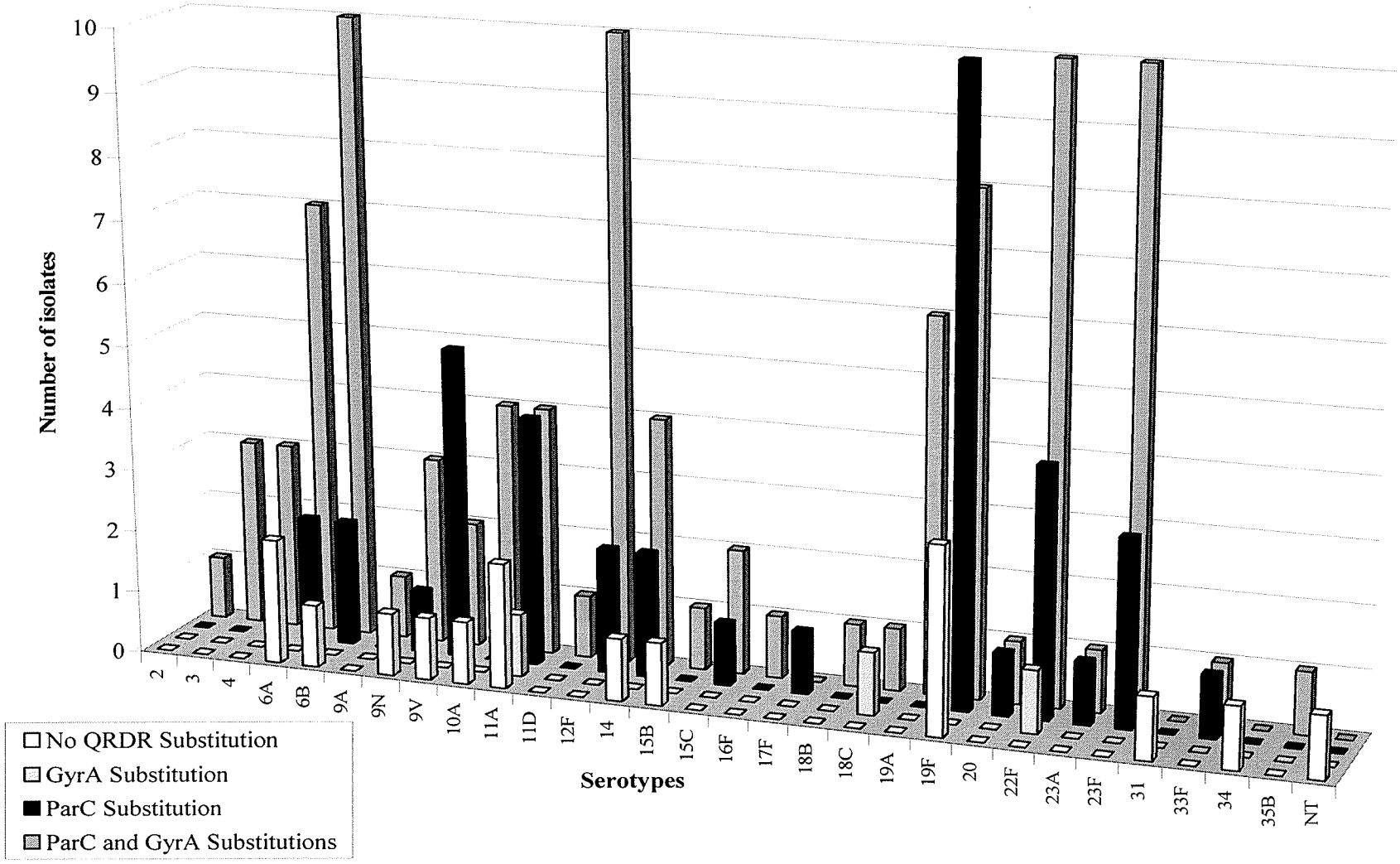

25
20
15
10
5
0
Number of isolates
6A
6B
9V
11A
12F
14
19A
19F
22F
23F
Serotypes
2004 (n=43)
03 (n=31)
01/02 (n=21)
00/01 (n=21)
99/00 (n=22)
98/99 (n=14)
97/98 (n=4)

Figure 3.9 **Serotypes of the 156 ciprofloxacin-resistant *S. pneumoniae* isolates based on the presence of substitutions in the QRDRs of ParC and/or GyrA. NT, non-typeable**

Fig. 3.9


Number of isolates
10
9
8
7
6
5
4
3
2
1
0
2
3
4
6A
6B
9A
9N
9V
10A
11A
11D
12F
14
15B
15C
16F
17F
18B
18C
19A
19F
20
22F
23A
23F
31
33F
34
35B
NT
Serotypes
No QRDR Substitution
GyrA Substitution
ParC Substitution
ParC and GyrA Substitutions

GyrA and ParC. Serotypes 31, 34, and non-typeable were observed in isolates with no QRDR substitutions. Serotype 17F was observed in an isolate with a ParC substitution. In contrast, serotype 11A was observed in isolates with no QRDR substitutions, single ParC substitutions, single GyrA substitutions, and substitutions in GyrA and ParC. Serotypes 6A, 6B, 9N, 9V, 14, and 19F were observed in isolates with no QRDR substitutions, single ParC substitutions, and substitutions in GyrA and ParC. Similarly, serotype 22F was associated with isolates possessing single ParC substitutions, single GyrA substitutions, and substitutions in GyrA and ParC.

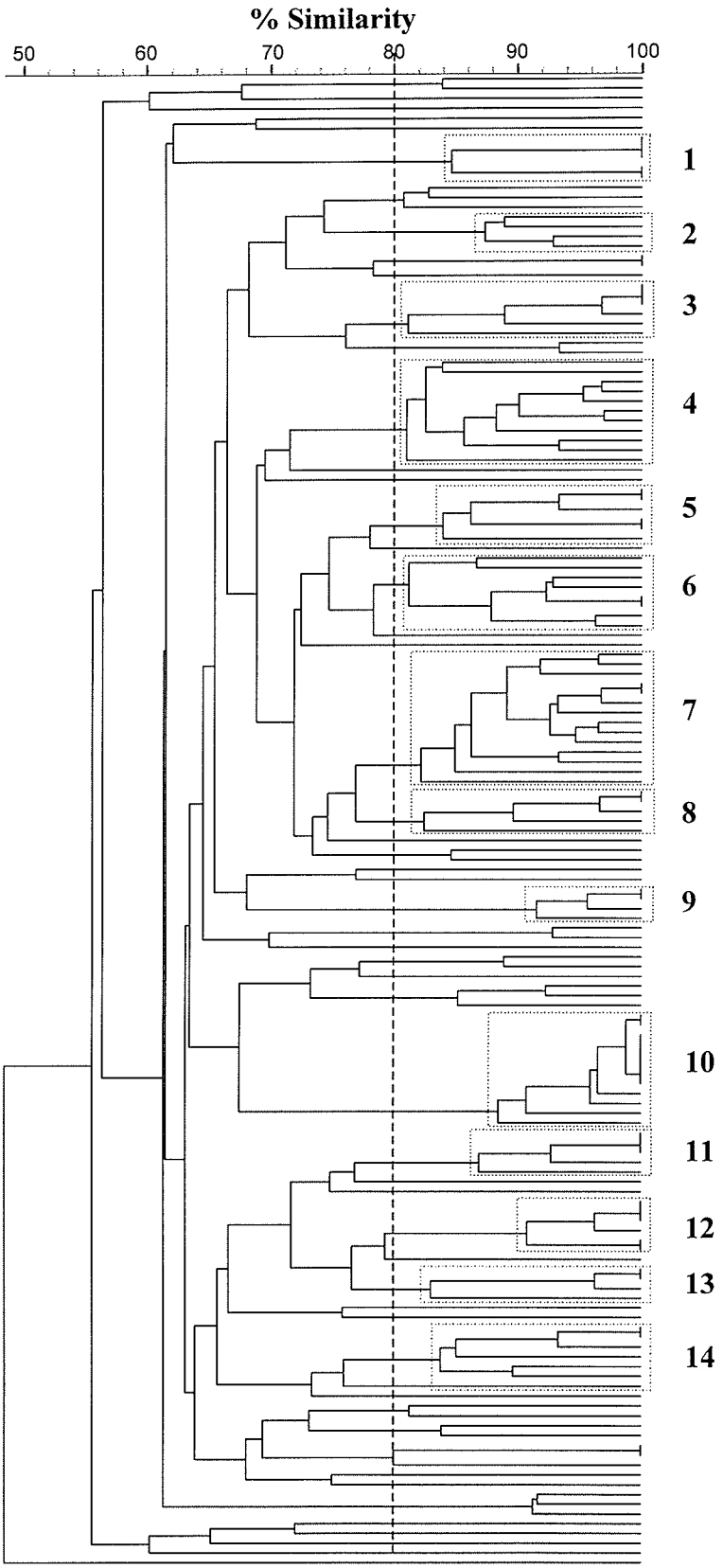
iv. Analysis of Molecular Epidemiology by PFGE

A dendrogram depicting the genetic relatedness of the ciprofloxacin-resistant *S. pneumoniae* isolates on the basis of PFGE results is presented in figure 3.10. There were 14 clusters, numbered 1 -14 on the figure, with 4 or more isolates per cluster observed among the isolates. Clusters 2, 9, and 13 each included 4 isolates. Clusters 1, 8, and 11 each had 5 isolates. Clusters 3, 5, 12, and 14 each consisted of 6 isolates. Clusters 4, 6, 7, and 10 included 11, 8, 14, and 12 isolates, respectively. The demographics, antibiotic susceptibilities and molecular characterization data for all the isolates were described in appendices A and B and table 3.1. Isolates were considered multi-drug resistant if they were resistant to penicillin and 2 other antimicrobials from different drug classes (107). In this study, multi-drug resistant isolates were resistant to ciprofloxacin, penicillin, and clarithromycin, doxycycline or trimethoprim-sulfamethoxazole.

Cluster 1 consists of isolates 56276, 56419, 56604, 29927, and 45776. The isolates were collected between 2001 and 2004 from geographically diverse regions. Four of the 5 isolates were serotype 10A and 1 was serotype 6B. All isolates had a ParC

Figure 3.10 **Dendrogram depicting genetic relatedness of the 156 ciprofloxacin-resistant *S. pneumoniae* isolates on the basis of PFGE results. PFGE was conducted with *Sma*I digestions. 80% similarity is indicated with a dashed line. Clusters are boxed with dotted lines and cluster number is indicated.**

Fig. 3.10



(Ser79Phe) substitution. Additionally, 2 isolates had a GyrA (Glu85Lys) substitution and the other 3 isolates had a GyrA (Ser81Phe) substitution.

Isolates 10280, 55719, 27224, and 52941 comprise cluster 2. The isolates were collected between 1998 and 2004 from Quebec and Saskatchewan. All isolates were serotype 6A. Three isolates had substitutions in GyrA and ParC (2 Ser81Phe and Ser79Phe and 1 Ser81Tyr and Ser79Phe). One isolate had a single substitution in ParC: Ser79Ala.

Cluster 3 includes isolates 47224, 47225, 50946, 47209, 19102, and 51126. The isolates were collected between 1999 and 2003 from across Canada. Five of the isolates were serotype 19F and the other was serotype 19A. Two isolates had substitutions in GyrA and ParC (Ser81Phe and Ser79Phe) while the other 4 isolates had a substitution in ParC (3 Ser79Phe and 1 Asp83Asn). Four of the isolates were multi-drug resistant.

Isolates 1282, 27908, 31685, 57278, 55374, 20336, 27917, 14769, 47396, 53482, and 24086 make up cluster 4. The isolates were collected between 1997 and 2004 from various regions of Canada. Seven isolates were serotype 9V, 3 were serotype 14, and 1 was serotype 9A. Four isolates had substitutions in GyrA and ParC (3 Ser81Phe and Ser79Phe, 1 Glu85Lys and Ser79Tyr) and 5 isolates had a substitution in ParC (4 Asp83Asn and 1 Ser79Phe). Ten of the isolates also had the ParC substitution Lys137Asn. Eight isolates were resistant to penicillin, seven of which were multi-drug resistant.

Cluster 5 consists of isolates 23335, 50770, 29516, 14033, 21181, and 55798. The isolates were collected from 3 provinces between 1998 and 2004. Five of the isolates were serotype 22F and 1 was serotype 6A. Five isolates had substitutions in

GyrA and ParC (1 Ser81Phe and Ser79Phe, 1 Ser81Phe and Ser79Tyr, 1 Ser81Phe and Ser79Tyr / Asp83Asn, 1 Ser81Tyr and Ser79Tyr, and 1 Glu85Lys and Ser79Phe).

Isolates 47797, 56319, 14744, 48486, 23786, 27546, 34604, 57155 comprise cluster 6. The isolates were collected between 1998 and 2004 from various provinces. All isolates were serotype 22F. Three isolates had substitutions in GyrA and ParC (1 Glu85Lys and Ser79Phe / Asp83Tyr, and 2 Ser81Phe and Ser79Tyr). One isolate had a substitution in GyrA (Ser81Phe) and four isolates had a ParC substitution (2 Ser79Tyr and 2 Ser79Phe).

Cluster 7 consists of isolates 10277, 11361, 55073, 12291, 12873, 52418, 33035, 4610, 44171, 51531, 18955, 56298, 22668, 45089. The isolates were collected between 1997 and 2004 from 8 provinces. Ten isolates were serotype 11A, and the other 4 were serotypes 11D, 19A, 31, and 33F. Five isolates had substitutions in GyrA and ParC (1 Ser81Phe and Ser79Phe, 1 Ser81Phe and Ser79Tyr, 1 Ser81Phe and Asp83Asn, 1 Glu85Lys and Ser79Phe, and 1 Ser81Phe and Asp78Ala). One isolate had a single GyrA substitution (Ser81Phe) and 5 isolates had a ParC substitution (3 Ser79Phe and 2 Ser79Tyr).

Isolates 25268, 32534, 54883, 45336, and 27833 were in cluster 8. The isolates were collected between 2000 and 2004 from Ontario and Quebec. Two isolates were serotype 18C, and the other 3 were serotypes 18B, 19A, and 33F. Four of the isolates had substitutions in GyrA and ParC (2 Ser81Phe and Ser79Phe, 1 Ser81Tyr and Ser79Tyr, and 1 Glu85Lys and Ser79Tyr). One isolate had a substitution in GyrA (Ser81Phe).

Cluster 9 is comprised of isolates 42372, 55666, 55660, and 56414. These isolates were collected between 2001 and 2004 from 3 provinces. All isolates were serotype 23F. Three isolates had substitutions in GyrA and ParC (1 Glu85Gly and Ser79Phe, 1 Glu85Lys and Ser79Phe, and 1 Ser81Phe and Ser79Phe) and 1 isolate had a substitution in ParC (Asp83Asn).

Isolates 48866, 49773, 48198, 48865, 49755, 49928, 55072, 55431, 35152, 54610, 53155, and 23574 form cluster 10. The isolates were collected between 1999 and 2004 from 3 provinces and all were serotype 12F. Ten of the isolates had substitutions in both GyrA and ParC (9 Ser81Phe and Ser79Phe and 1 Ser81Tyr and Ser79Phe). Two isolates had a ParC substitution (Ser79Phe). All isolates had the Lys137Asn substitution in ParC.

Cluster 11 consists of isolates 45693, 49710, 49711, 33726, and 53683. The isolates were collected between 2001 and 2004 from 3 provinces. Three isolates were serotype 3 and 2 were 9N. All isolates had substitutions in GyrA and ParC (4 Ser81Phe and Ser79Phe and 1 Ser81Phe and Ser79Tyr).

Isolates 26608, 34547, 34549, 50227, 16072, and 39710 are in cluster 12. The isolates were collected from 4 provinces between 1999 and 2003. One isolate was serotype 2, 2 were 15C, 1 was 15B, and 2 were 19F. Four of the isolates had substitutions in GyrA and ParC (2 Ser81Phe and Ser79Phe, 1 Ser81Phe and Ser79Tyr, and 1 Glu85Lys and Asp83Asn).

Cluster 13 is comprised of isolates 49322, 56283, 29228, and 9989. The isolates were collected between 1997 and 2004 in 3 provinces. Two isolates were serotype 19A

and the other 2 were 19F and 23F. All isolates had substitutions in ParC and GyrA (3 Ser81Phe and Ser79Phe and 1 Ser81Phe and Asp83Tyr).

Isolates 11434, 14904, 49101, 56904, 29111, and 45333 make up cluster 14. The isolates were collected between 1998 and 2004 from various provinces. Four isolates were serotype 23F and the other 2 were serotype 19F. Five isolates had substitutions in GyrA and ParC (4 Ser81Phe and Ser79Phe and 1 Glu85Lys and Ser79Phe). One isolate had a Ser79Phe substitution in ParC. All isolates had the ParC Lys137Asn substitution. Two isolates were multi-drug resistant.

d. Full gene sequencing of *gyrA*, *parC*, and *parE*

i. Isolate Selection

Sixty isolates were selected for full gene sequencing analysis of *gyrA*, *parC*, and *parE*. Six fluoroquinolone-susceptible isolates (2587, 2663, 2670, 46253, 47805, and 49728) that had been shown previously to have no QRDR substitutions in GyrA or ParC and were negative for reserpine-inhibited efflux were included as controls. These isolates are included in the studies described in results parts II and III. Fifty-four ciprofloxacin-resistant isolates of the 156 aforementioned ciprofloxacin-resistant isolates were randomly selected such that isolates without QRDR substitutions in GyrA or ParC (n=10), isolates with GyrA QRDR substitutions (n=3), isolates with ParC QRDR substitutions (n=17), and isolates with QRDR substitutions in both GyrA and ParC (n=24) were included in the study. Isolates were collected from 7 provinces and represented 22 serotypes.

ii. Amplification of *gyrA*, *parC*, and *parE*

Figure 3.11 presents the amplification products of *gyrA*, *parC*, and *parE* using the primers designed in this study and listed in table 2.2. A 1Kb plus ladder was included on the gel for product size determination. The products were of the expected size: *gyrA* (2.6Kb), *parC* (2.7Kb), and *parE* (2.4Kb). The similar size of the amplification products of *gyrA* and *parC* is expected as these genes have high homology and code for proteins that ultimately carry out comparable cellular functions.

iii. Sequencing of *gyrA*, *parC*, and *parE*

The ciprofloxacin MICs, QRDR substitutions and non-QRDR substitutions of all sixty isolates are presented in table 3.2. The non-QRDR substitutions observed in the fluoroquinolone-susceptible isolates included Val489Ile and Ala653Thr in GyrA, Lys246Asn, Ala450Val, Glu589Ala, Val608Ala, and Asp822Tyr in ParC, and Ile162Val and Tyr263His in ParE.

The non-QRDR GyrA substitutions observed in the ciprofloxacin-resistant isolates included Ala191Val, Ser237Leu, Arg295His, Ser418Thr, Val486Ile, Val489Ile, Glu560Asp, Ala653Thr, Ile711Val, Leu747Phe, Val768Phe, and Ser778Leu. The ParC non-QRDR substitutions observed in the ciprofloxacin-resistant isolates were Ala189Val, Thr257Ile, Ala282Thr, Asp294Leu, His373Arg, Ala394Thr, Arg443Cys, Ala450Val, Ile453Tyr, Lys473Asn, Thr493Ile, Arg518His, Thr582Ile, Arg569Cys, Glu589Ala, Val608Ala/Ser, Met686Ile, Ala724Ser, and Asp822Tyr. ParE non-QRDR substitutions observed in ciprofloxacin-resistant isolates included Gly34Arg, Ser132Asn, Ile162Val, Pro166Leu, Ser200Leu, Thr212Ala, Thr216Ser, Asp217Asn, Leu290Phe, Ala326Val, Val355Ile, Gly372Arg, Leu374Ile, Ser399Ile, and Ala644Thr. The percent of

Figure 3.11 ***gyrA*, *parC*, and *parE* amplification products were run on a 1% agarose gel and visualized by ethidium bromide staining. Lanes 1 and 14 are 1 Kb plus ladders. Lanes 2 – 5 are the *gyrA* products. Lanes 6 – 9 are the *parC* products. Lanes 10 – 13 are *parE* products. Lanes 2, 6, and 10 are products of isolate 29228. Lanes 3, 7, and 11 are products of isolates 46253. Lanes 4, 8, and 12 are products of isolates 47805. Lanes 5, 9, and 13 are products of isolate 49728.**

Fig. 3.11

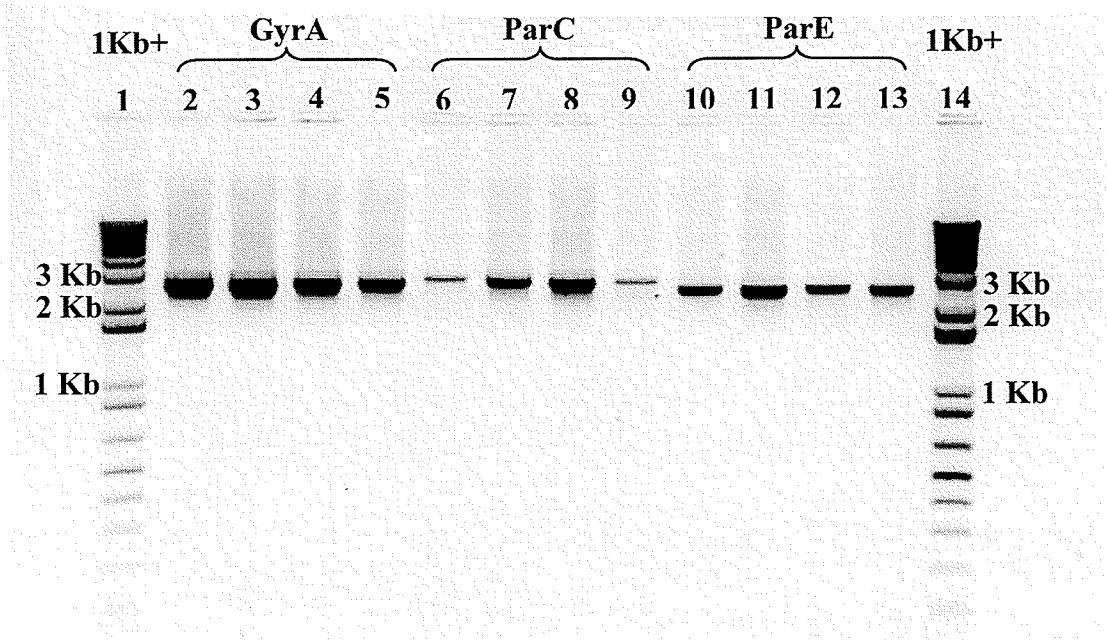

1Kb+
GyrA
ParC
ParE
1Kb+
1 2 3 4 5 6 7 8 9 10 11 12 13 14
3 Kb
2 Kb
1 Kb
3 Kb
2 Kb
1 Kb

Table 3.2 **Ciprofloxacin MICs and QRDR and non-QRDR substitutions observed in GyrA, ParC, and ParE in fluoroquinolone-susceptible and ciprofloxacin-resistant *S. pneumoniae* isolates**

Isolate	Cipro (μg/mL)	GyrA		ParC		ParE		Efflux
		QRDR	Non-QRDR	QRDR	Non-QRDR	QRDR	Non-QRDR	
Fluoroquinolone-susceptible								
2587	**1**	None	Val489Ile	None	Asp822Tyr	None	None	Neg.
2663	**1**	None	Val489Ile	None	Asp822Tyr	None	None	Neg.
2670	**1**	None	Val489Ile	None	Asp822Tyr	None	None	Neg.
46253	**1**	None	Val489Ile	None	Lys246Asn	Ile460Val	Ile162Val, Tyr263His	Neg.
47805	**0.5**	None	Val489Ile, Ala653Thr	None	Ala450Val, Glu589Ala, Val608Ala	Ile460Val	None	Neg.
49728	**1**	None	Val489Ile, Ala653Thr	None	Ala450Val, Glu589Ala, Val608Ala	Ile460Val	None	Neg.
Ciprofloxacin-resistant								
984	**8**	Ser81Phe	Val486Ile	Ser79Phe	None	None	Ile162Val	Neg.
1146	**8**	Ser81Phe	Val489Ile	None	Asp822Tyr	Asp435Asn	None	Neg.
1282	**8**	None	Val489Ile	Lys137Asn	Val608Ala	Il460Val, Glu474Lys	None	Pos.
4610	**4**	None	Val489Ile	Ser79Phe	Ala450Val, Glu589Ala, Val608Ala	Ile460Val	None	Neg.
10277	**8**	None	Val489Ile, Ala653Thr	Ser79Phe	Ala450Val	Ile460Val	None	Pos.
10733	**16**	None	Val486Ile	Ser79Phe	None	None	Ile162Val	Neg.

11434	**4**	None	Val489Ile, Ala653Thr	Ser79Phe, Lys137Asn	None	Ile460Val	Ser132Asn, Leu290Phe, Ala326Val	Neg.
12291	**4**	None	Val489Ile, Ala653Thr	Ser79Tyr	Ala450Val, Glu589Ala, Val608Ala	Ile460Val	None	Neg.
12873	**8**	None	Val489Ile, Ala653Thr	Ser79Phe	Ala450Val	Ile460Val	None	Pos.
14744	**4**	None	Val489Ile, Ala653Thr	Ser79Phe	Val608Ala	None	Ile162Val, Thr216Ser	Neg.
14769	**8**	None	Val489Ile	Ser79Phe, Lys137Asn	Val608Ala	Ile460Val	None	Neg.
15017	**4**	None	Val489Ile, Ala653Thr	None	Ala394Thr	Ile460Val	Gly34Arg	Pos.
16072	**4**	None	Val489Ile, Ala653Thr	None	His373Arg, Glu589Ala, Lys473Asn, Val608Ala	Ile460Val	None	Pos.
17012	**16**	Ser81Phe	None	Ser79Phe	His373Arg, Lys473Asn, Glu589Ala, Val608Ala	None	Pro166Leu	Neg.
17913	**8**	None	Ser237Leu, Val486Ile	Asp83Gly	Asp294Leu, Ile453Tyr, Val608Ser	None	Asp217Asn	Pos.
19103	**4**	None	Val489Ile	None	Ala282Thr, Val608Ala	Asp435Asn, Ile460Val	None	Neg.
19120	**16**	None	Val486Ile, Ser778Leu	Asp83Asn	None	Ile460Val	None	Pos.

20336	**8**	None	Val489Ile	Asp83Asn, Lys137Asn	Val608Ala	None	None	Neg.
21181	**32**	Glu85Lys	Val489Ile, Ala653Thr	Ser79Phe	Val608Ala	None	Ile162Val, Thr216Ser	Neg.
22360	**8**	None	Val489Ile	Asp83Ala, Lys137Asn	Val608Ala	Ile460Val	None	Neg.
22627	**8**	None	Val768Phe	Asp83Gly	Ala189Val, Val608Ala	Ile460Val	Pro166Leu, Thr212Ala	Neg.
23335	**32**	Ser81Phe	Val489Ile, Ala653Thr	Ser79Phe	Val608Ala	None	Ile162Val, Thr216Ser	Neg.
23574	**32**	Ser81Phe	Ile711Val	Ser79Phe, Lys137Asn	None	Ile460Val	None	Neg.
23786	**4**	Ser81Phe	Val489Ile, Ala653Thr	None	Val608Ala	Asp435Asn	Ile162Val, Thr216Ser	Neg.
24086	**8**	None	Val489Ile	Tyr59Asp, Lys137Asn	Val608Ala	Ile460Val	Ser399Ile	Neg.
25074	**4**	None	None	Ser79Phe	Thr582Ile	None	None	Neg.
25268	**16**	Ser81Phe	None	None	Ala450Val, Arg518His, Glu589Ala, Val608Ala	Asp435Asn, Ile460Val	None	Neg.
28669	**4**	None	Val486Ile	Ser107Tyr	None	None	Ile162Val	Neg.
29012	**4**	None	Val486Ile	None	Ala189Val, Threo493Ile, Val608Ala	None	Ile162Val	Pos.
29228	**32**	Ser81Phe	Val489Ile, Glu560Asp, Leu747Phe	Asp83Tyr	Thr257Ile, Asp822Tyr	Ile460Val, Glu474Lys	Asp217Asn, Ala644Thr	Neg.
30890	**4**	None	Val486Ile	Lys137Asn	Arg569Cys	None	Ser200Leu	Pos.

30900	**4**	Ser81Phe	None	Ser79Phe	None	Ile460Val	Ser132Asn, Leu374Ile	Neg.
32534	**16**	Ser81Tyr	Val486Ile	Ser79Tyr	Ala394Thr	Ile460Val	Gly372Arg	Neg.
32839	**8**	Ser81Phe	Val486Ile	Ser79Tyr	None	None	Ile162Val	Neg.
33035	**16**	Ser81Phe	Val489Ile, Ala653Thr	None	Ala450Val, Glu589Ala, Val608Ala	Ile460Val	None	Neg.
34549	**16**	Ser81Phe	Val489Ile, Ala653Thr	Ser79Phe	Ala394Tyr	Ile460Val	None	Neg.
35097	**8**	Ser81Tyr	Val489Ile	Ser79Phe	Asp822Tyr	None	None	Neg.
39710	**8**	Ser81Phe	Val489Ile	Ser79Phe	His373Arg, Lys473Asn, Glu589Ala, Val608Ala	Asp435Asn, Ile460Val	None	Pos.
44171	**4**	None	Val489Ile, Ala653Thr	None	Ala450Val, Glu589Ala, Val608Ala	Ile460Val	None	Pos.
45089	**4**	None	Val486Ile	None	His373Arg, Arg443Cys, Glu589Ala, Val608Ala, Asp822Tyr	Ile460Val	Ser132Asn, Leu290Phe	Pos.
45693	**16**	Ser81Phe	Ser418Thr	Ser79Phe	None	Ile460Val	None	Pos.
45776	**8**	Ser81Tyr	Ala191Val, Arg295His, Val489Ile	Ser79Phe	Val608Ala	None	None	Neg.
45780	**16**	None	Val486Ile	Ser79Tyr	None	None	None	Neg.

47797	**4**	Glu85Lys	Val489Ile, Ala653Thr	Ser79Phe, Asp83Tyr	Val608Ala	None	Thr216Ser	Neg.
49322	**4**	Ser81Phe	Val489Ile, Ala653The	Ser79Phe	Val608Ala	None	Ile162Val, Thr216Ser	Neg.
49711	**8**	Ser81Phe	None	Ser79Phe	Ala450Val, Glu589Ala, Val608Ala	Ile460Val	None	Pos.
51597	**4**	None	Val489Ile, Ala653Thr	Ser79Phe	None	Ile460Val	Val355Ile	Pos.
52418	**32**	Ser81Phe	Val489Ile, Ala653Thr	Ser79Tyr, Lys137Asn	His373Arg, Arg443Cys, Glu589Ala, Val608Ala	Ile460Val	None	Neg.
53155	**8**	Ser81Phe	Ile711Val	Ser79Phe, Lys137Asn	Ala724Ser, Asp822Tyr	None	None	Neg.
53482	**16**	Ser81Phe	Val489Ile	Ser79Phe, Lys137Asn	Val608Ala, Met686Ile	Ile460Val	None	Neg.
54610	**4**	Ser81Phe	Ile711Val	Ser79Phe, Lys137Asn	Ala724Ser, Asp822Tyr	None	None	Neg.
55160	**8**	Ser81Phe	Val489Ile	Ser79Phe	Asp822Tyr	Asp435Asn	None	Neg.
55178	**8**	Ser81Phe	Val489Ile	Ser79Phe	None	None	None	Neg.
55430	**16**	Ser81Tyr	Ile711Val	Ser79Phe, Lys137Asn	Ala724Ser, Asp822Tyr	None	None	Neg.

Cipro, ciprofloxacin; Neg., negative; Pos., positive

ciprofloxacin-resistant isolates with each non-QRDR substitution observed in GyrA, ParC, and ParE is presented in figures 3.12a –c.

All 16 isolates with an Ala653Thr substitution in GyrA also had a Val489Ile substitution in GyrA. These isolates were collected from 7 provinces and represented 9 serotypes. The 11 isolates with a Glu589Ala substitution in ParC also had a Val608Ala substitution in ParC. These isolates originated from 5 provinces and included 8 serotypes. All 6 isolates with a Thr216Ser substitution in ParE also had a Val608Ala substitution in ParC and Val489Ile and Ala653Thr substitutions in GyrA. The isolates were received from 4 provinces and included 2 serotypes. The 5 isolates with a His373Arg substitution in ParC also had Glu589Ala and Val608Ala ParC substitutions. These isolates were collected from 3 provinces and represented 3 serotypes. The 3 isolates with a ParC Lys473Asn substitution also had His373Arg, Glu589Ala, and Val608Ala substitutions in ParC. These isolates were received from 2 provinces. The isolates were all serotype 19F. Two of the isolates were identical by PFGE and 1 was unrelated. The 3 isolates with an Ala724Ser substitution in ParC also had an Asp822Tyr substitution in ParC and an Ile711Val substitution in GyrA. The isolates originated from Manitoba and included 2 serotypes. Two of the isolates clustered by PFGE and 1 was unrelated. Apart from the 2 cases mentioned, the isolates described herein were not found to cluster by PFGE.

Overall, of the 54 ciprofloxacin-resistant isolates, 91% had non-QRDR substitutions in GyrA, 78% had non-QRDR substitutions in ParC, and 35% had non-QRDR substitutions in ParE.

Figure 3.12 **Percent of 54 ciprofloxacin-resistant isolates with non-QRDR substitutions in (a) GyrA, (b) ParC, and (c) ParE. Number of isolates with each substitution is indicated in parentheses.**

Fig. 3.12a

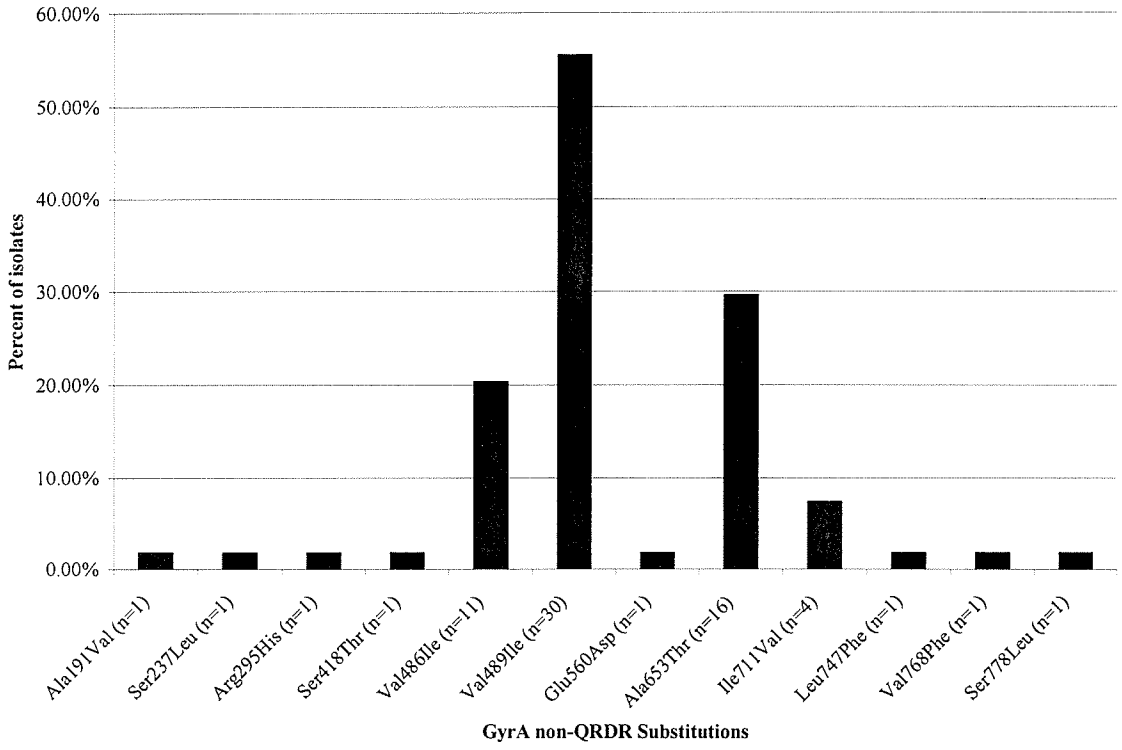

60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
Percent of isolates
Ala191Val (n=1)
Ser237Leu (n=1)
Arg295His (n=1)
Ser418Thr (n=1)
Val486Ile (n=11)
Val489Ile (n=30)
Glu560Asp (n=1)
Ala653Thr (n=16)
Ile711Val (n=4)
Leu747Phe (n=1)
Val768Phe (n=1)
Ser778Leu (n=1)
GyrA non-QRDR Substitutions

Fig. 3.12b

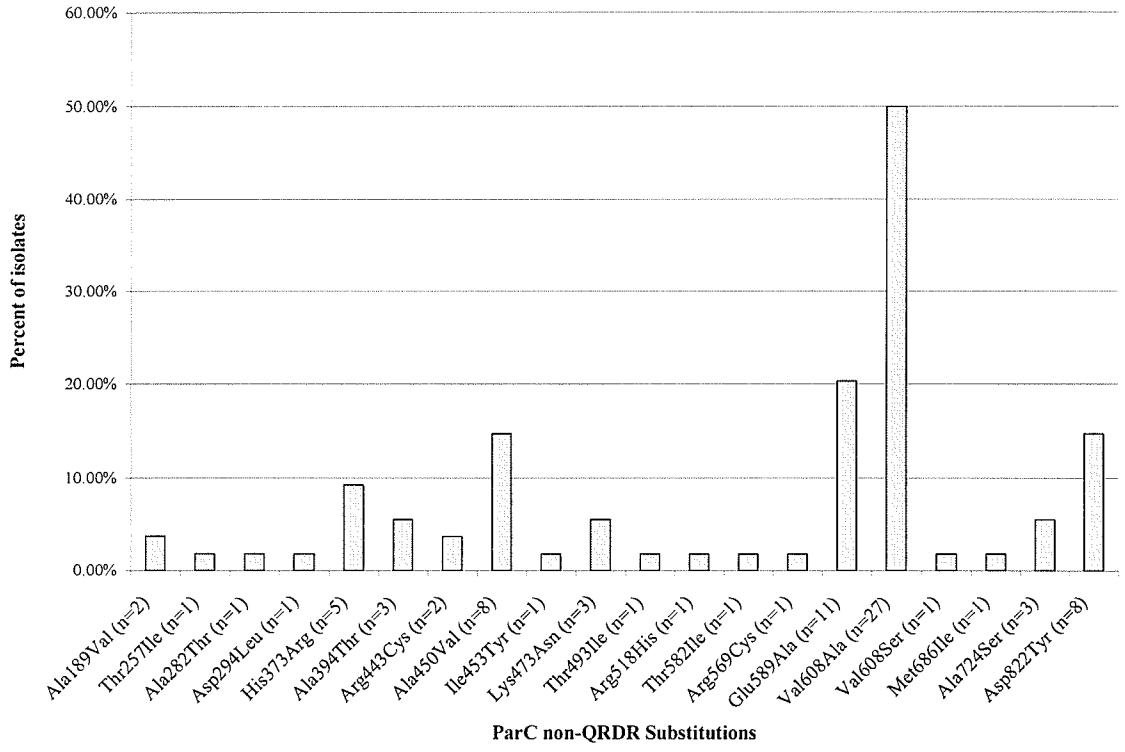
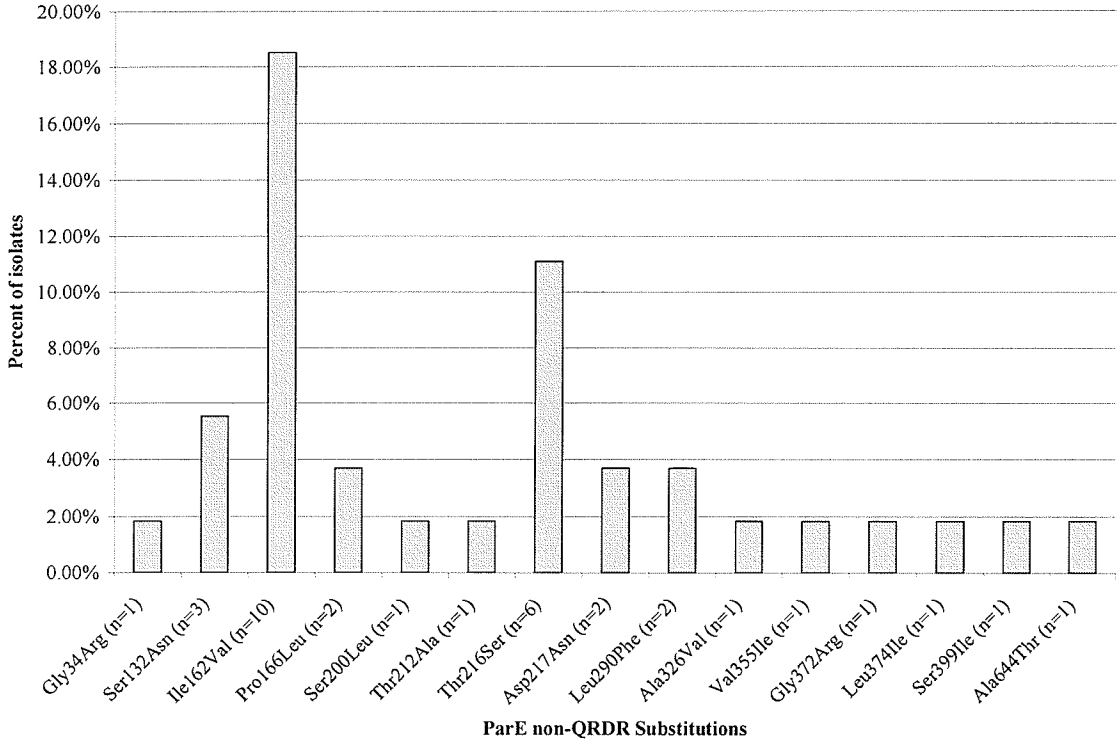

60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
Percent of isolates
Ala189Val (n=2)
Thr257Ile (n=1)
Ala282Thr (n=1)
Asp294Leu (n=1)
His373Arg (n=5)
Ala394Thr (n=3)
Arg443Cys (n=2)
Ala450Val (n=8)
Ile453Tyr (n=1)
Lys473Asn (n=3)
Thr493Ile (n=1)
Arg518His (n=1)
Thr582Ile (n=1)
Arg569Cys (n=1)
Glu589Ala (n=11)
Val608Ala (n=27)
Val608Ser (n=1)
Met686Ile (n=1)
Ala724Ser (n=3)
Asp822Tyr (n=8)
ParC non-QRDR Substitutions

Fig. 3.12c


20.00%
18.00%
16.00%
14.00%
12.00%
10.00%
8.00%
6.00%
4.00%
2.00%
0.00%
Percent of isolates
Gly34Arg (n=1)
Ser132Asn (n=3)
Ile162Val (n=10)
Pro166Leu (n=2)
Ser200Leu (n=1)
Thr212Ala (n=1)
Thr216Ser (n=6)
Asp217Asn (n=2)
Leu290Phe (n=2)
Ala326Val (n=1)
Val355Ile (n=1)
Gly372Arg (n=1)
Leu374Ile (n=1)
Ser399Ile (n=1)
Ala644Thr (n=1)
ParE non-QRDR Substitutions

The percent of ciprofloxacin-resistant isolates with non-QRDR substitutions in GyrA, ParC, and ParE is presented in figure 3.13a based on the ciprofloxacin MIC of the isolates. At a ciprofloxacin MIC of 4 μg/mL (n=18), 89%, 78%, and 67% of the isolates had non-QRDR substitutions in GyrA, ParC, and ParE, respectively. Ninety-four percent, 83%, and 39% of the isolates with a ciprofloxacin MIC of 8 μg/mL (n=18) had non-QRDR substitutions in GyrA, ParC, and ParE, respectively. For the isolates with a ciprofloxacin MIC of 16 μg/mL (n=13), 87% had GyrA non-QRDR substitutions, 62% had ParC non-QRDR substitutions, and 31% had ParE non-QRDR substitutions. At a ciprofloxacin MIC of 32 μg/mL (n=5), 100%, 100%, and 60% of the isolates had non-QRDR substitutions in GyrA, ParC, and ParE, respectively.

The percent of isolates with non-QRDR substitutions in GyrA, ParC, and ParE is presented in figure 3.13b based on the presence of QRDR substitutions. In isolates without QRDR substitutions associated with resistance (n=7), 100%, 100%, and 57% had GyrA, ParC, and ParE non-QRDR substitutions, respectively. Ninety-four percent, 67%, and 56% of the isolates with a single ParC QRDR substitution (n=18) had non-QRDR substitutions in GyrA, ParC, and ParE, respectively. In the isolates with QRDR substitutions in GyrA and ParC (n=23), 87%, 74%, and 48% had non-QRDR substitutions in GyrA, ParC, and ParE, respectively. In the isolates with ParC and ParE QRDR substitutions (n=2), 100% had non-QRDR substitutions in GyrA, 100% had non-QRDR substitutions in ParC, and 0% had non-QRDR substitutions in ParE. Sixty-seven percent, 100%, and 33% of the isolates with QRDR substitutions in GyrA and ParE (n=3) had non-QRDR substitutions in GyrA, ParC, and ParE, respectively. In the isolates with

Figure 3.13 **Percent of ciprofloxacin-resistant isolates with non-QRDR substitutions in GyrA, ParC, and ParE based on (a) ciprofloxacin MIC and (b) QRDR substitutions in GyrA, ParC, and ParE. The number of isolates in each category: MIC or QRDR substitution, are indicated in parentheses.**

Fig. 3.13a

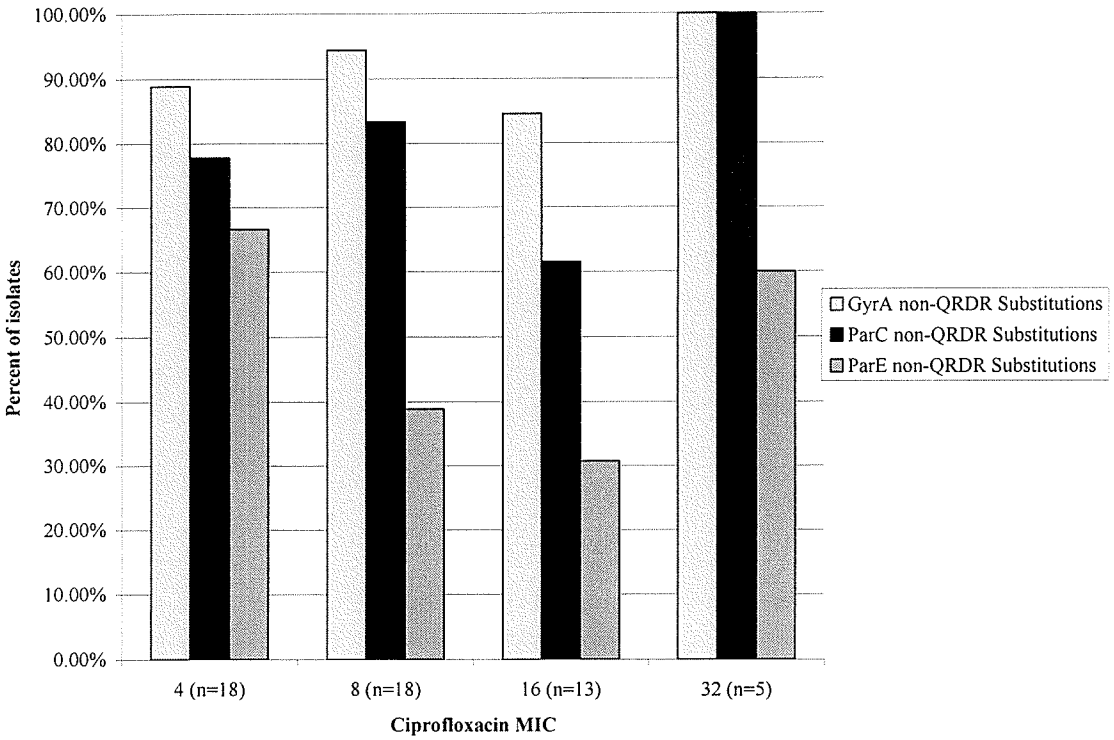
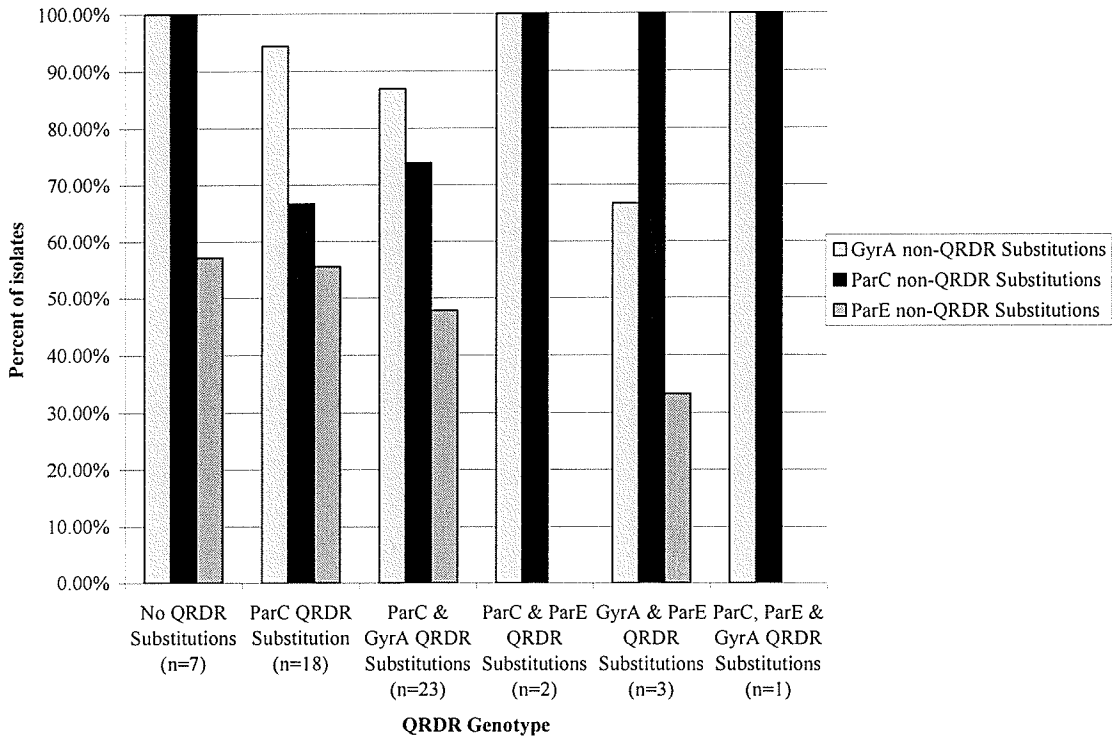

100.00%
90.00%
80.00%
70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
Percent of isolates
4 (n=18)
8 (n=18)
16 (n=13)
32 (n=5)
Ciprofloxacin MIC
GyrA non-QRDR Substitutions
ParC non-QRDR Substitutions
ParE non-QRDR Substitutions

Fig. 3.13b


100.00%
90.00%
80.00%
70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
Percent of isolates
GyrA non-QRDR Substitutions
ParC non-QRDR Substitutions
ParE non-QRDR Substitutions
No QRDR Substitutions (n=7)
ParC QRDR Substitution (n=18)
ParC & GyrA QRDR Substitutions (n=23)
ParC & ParE QRDR Substitutions (n=2)
GyrA & ParE QRDR Substitutions (n=3)
ParC, ParE & GyrA QRDR Substitutions (n=1)
QRDR Genotype

GyrA, ParC, and ParE QRDR substitutions (n=1), 100%, 100%, and 0% had non-QRDR substitutions in GyrA, ParC, and ParE, respectively.

Part II: Analysis of Fluoroquinolone Resistance-Associated Substitutions in Canadian Clinical Fluoroquinolone-Susceptible *S. pneumoniae* Isolates

a. Demographics

Over 900 fluoroquinolone-susceptible isolates were evaluated in the following studies. The isolates were collected as part of CROSS from 9 Canadian provinces: Alberta (15%), British Columbia (12%), Manitoba (14%), New Brunswick (4%), Nova Scotia (4%), Ontario (23%), Prince Edward Island (1%), Quebec (16%), and Saskatchewan (11%). Sixty-four percent of isolates were from males and 36% from females. Thirteen percent of isolates were from children (<16 years of age), 45% were from adults (16-64 years of age), and 42% were from elderly patients (>64 years of age).

b. Determination of Genetic-based Fluoroquinolone Breakpoints

The study determining microbiological resistance breakpoints for gatifloxacin, gemifloxacin, levofloxacin, and moxifloxacin has been published (121).

i. Isolate Selection

Thirty-eight ciprofloxacin-resistant isolates described in part I that were susceptible to gatifloxacin, gemifloxacin, levofloxacin, and moxifloxacin were included in this study. Additionally, 116 fluoroquinolone-susceptible *S. pneumoniae* isolates were selected to include MICs at or lower than the current susceptible breakpoint, encompass all Canadian geographic regions, and incorporate all years of CROSS (1997/98 - 2003) with an emphasis on 2003.

ii. Sequencing of the Quinolone Resistance-Determining Regions of *gyrA* and *parC*

Data for the 154 isolates studied: year and location of isolation, MICs of ciprofloxacin, gatifloxacin, gemifloxacin, levofloxacin, and moxifloxacin, and the results of the QRDR sequencing are included in appendix C. For gatifloxacin susceptible isolates (MIC ≤ 1 μg/mL), 25% of the isolates (n=141) had ParC substitutions, 2% had GyrA substitutions, 0% had substitutions in both ParC and GyrA, and 73% had no QRDR substitutions. For gemifloxacin susceptible isolates (MIC ≤ 0.12 μg/mL) (n=140), 24% had ParC substitutions, 2% had GyrA substitutions, 1% had substitutions in both ParC and GyrA, and 73% had no QRDR substitutions. For levofloxacin-susceptible (MIC ≤ 2 μg/mL), 23% of the isolates (n=154) had ParC substitutions, 2% had GyrA substitutions, 1% had both ParC and GyrA substitutions, and 73% of the isolates had no QRDR substitutions. For moxifloxacin susceptible-isolates (MIC ≤ 1 μg/mL), 24% of the isolates (n=153) had ParC substitutions, 2% had GyrA substitutions, 1% had substitutions in both ParC and GyrA, and 74% had no QRDR substitutions.

For ciprofloxacin-susceptible isolates (MIC ≤ 2 μg/mL) with a gatifloxacin MIC of ≤ 1 μg/mL (n=105), gemifloxacin MIC of ≤ 0.12 μg/mL (n=105), levofloxacin MIC of ≤ 2 μg/mL (n=116) or moxifloxacin MIC of ≤ 1 μg/mL (n=116) (susceptible by CLSI breakpoints), 9% of isolates had ParC QRDR substitutions and 2% had QRDR substitutions in GyrA.

However, for ciprofloxacin-resistant isolates (MIC ≥ 4 μg/mL) with a gatifloxacin MIC of ≤ 1 μg/mL (n=36), gemifloxacin MIC of ≤ 0.12 μg/mL (n=35), levofloxacin MIC of ≤ 2 μg/mL (n=38) or moxifloxacin MIC of ≤ 1 μg/mL (n=37) (susceptible by CLSI

breakpoints), 72, 71, 68, and 70% of isolates had substitutions in the QRDR of ParC, 3, 3, 3, and 3% had substitutions in the QRDR of GyrA, and 0, 3, 5, and 3% had substitutions in both ParC and GyrA, respectively.

iii. Determination of microbiological resistance breakpoints

The isolates were separated into three categories: few QRDR substitutions (< 15% of isolates), likely QRDR substitutions (15% to 60%, except levofloxacin), and very likely QRDR substitutions (> 60% of isolates) in order to establish microbiological breakpoints. Based on the proposed few QRDR substitutions category, 91, 94, 86, and 91% of the isolates had no QRDR substitutions, 8, 6, 14, and 8% had ParC substitutions and 1, 0, 0, and 1% had GyrA substitutions for gatifloxacin, gemifloxacin, levofloxacin, and moxifloxacin, respectively. In the proposed likely QRDR substitutions category, 49, 62, 96, and 49% had no QRDR substitutions, 49, 34, 3, and 49% had ParC substitutions, and 2, 4, 1, and 2% had GyrA substitutions for gatifloxacin, gemifloxacin, levofloxacin, and moxifloxacin, respectively. In the proposed very likely QRDR substitutions category, 20, 29, 38, and 25% had no QRDR substitutions, 50, 62, 55, and 38% had ParC substitutions, 10, 5, 4, and 13% had GyrA substitutions, and 20, 5, 4, and 25% had substitutions in GyrA and ParC for gatifloxacin, gemifloxacin, levofloxacin, and moxifloxacin, respectively.

The MICs of gatifloxacin, gemifloxacin, levofloxacin, and moxifloxacin corresponding to the few QRDR substitutions, likely QRDR substitutions, and very likely QRDR substitutions categories and the percent of isolates with QRDR substitutions at that MIC are presented in table 3.3. The proposed microbiological resistance breakpoint is defined as the MIC at which > 50% of the isolates carry QRDR substitutions. The

proposed resistance microbiologic breakpoints are (μg/mL): gatifloxacin > 0.25, gemifloxacin > 0.03, levofloxacin > 1, and moxifloxacin > 0.12, as presented in table 3.3.

Table 3.3 Current PK/PD breakpoints and proposed microbiological resistance breakpoints for fluoroquinolones and *S. pneumoniae.*

Fluoroquinolone	Current PK/PD BP	MIC μg/mL (% with substitutions)			Microbiological Resistant Breakpoint
		Few QRDR sub.	Likely QRDR sub.	Very likely QRDR sub.	
Gatifloxacin	1,2,4	≤0.25 (9%)	0.5 (51%)	≥1 (80%)	>0.25
Gemifloxacin	0.12, 0.25,0.5	≤0.015 (6%)	0.03 (38%)	≥0.06 (71%)	>0.03
Levofloxacin	2,4,8	≤0.5 (14%)	1 (4%)	≥2 (63%)	>1
Moxifloxacin	1,2,4	≤0.12 (9%)	0.25 (51%)	≥0.5 (75%)	>0.12

PK/PD, pharmacokinetic/pharmacodynamic; BP, breakpoint; sub., substitutions

c. Prevalence of Fluoroquinolone Resistance-Associated Substitutions in Fluoroquinolone-Susceptible *S. pneumoniae* Isolates Pre- and Post-introduction of Respiratory Fluoroquinolone Use in Canada

The prevalence of fluoroquinolone resistance-associated substitutions in fluoroquinolone-susceptible isolates between 1995/97 and 2003 have been published (112).

i. Isolate Selection

All fluoroquinolone-susceptible *S. pneumoniae* isolates with a levofloxacin MIC of 1 μg/mL collected as part of CROSS in 2003 (n = 665) were included in this study. Similarly, all fluoroquinolone-susceptible *S. pneumoniae* isolates with a levofloxacin

MIC of 1 μg/mL collected between 1995 and 1997 (n = 111) as part of CROSS and earlier studies were included. In total, 776 isolates were evaluated.

ii. Sequencing of the Quinolone Resistance-Determining Regions of *gyrA* and *parC*

Of the 111 isolates collected between 1995 and 1997, 30 (27%) had substitutions in the QRDR of ParC. The observed substitutions and the number of isolates possessing these substitutions, in parentheses, were: Ser52Gly and Lys137Asn (1), Ser79Phe (1), Arg95Cys (1), and Lys137Asn (27). Similarly, 161 of the 665 (24.2%) isolates collected in 2003 had substitutions in ParC or ParC and GyrA. The observed substitutions and the number of isolates possessing these substitutions, in parentheses, were: Ser52Asn (1), Ser52Gly and Lys137Asn (9), Ser79Phe (7), Ser79Phe and Ser81Phe (GyrA) (1), Ser79Phe and Lys137Asn (2), Ser79Tyr (3), Asp83Asn (1), Tyr129Ser (3), Glu135Asp (1), Lys137Asn (132), and Lys137Asn and Tyr98Asp (GyrA) (1).

Considering only QRDR substitutions known to confer resistance (101), 1 of the 111 (0.9%) isolates collected between 1995 and 1997 and 14 of the 665 (2.1%) isolates collected in 2003 had QRDR substitutions. An increase from 0.9% to 2.1% is statistically non-significant (p=0.34). The number of isolates with fluoroquinolone-resistance-associated QRDR amino acid substitutions and the specific substitutions observed are presented in table 3.4.

Table 3.4 **Number of fluoroquinolone-susceptible *S. pneumoniae* isolates (with levofloxacin MICs of 1 μg/mL) between 1995-1997 and in 2003 possessing fluoroquinolone resistance-associated QRDR substitutions in ParC and GyrA.**

Observed Substitutions		Number of Substitutions by Study Years	
ParC	**GyrA**	**1995-1997 (n=111)**	**2003 (n=665)**
Ser79Phe	None observed	1	9
Ser79Phe	Ser81Phe	0	1
Ser79Tyr	None observed	0	3
Asp83Asn	None observed	0	1
Total Number (%)		1 (0.9%)	14 (2.1%)

Part III: Laboratory-Selected Single-Step Fluoroquinolone-Resistant *S. pneumoniae* Mutants

The determination of the mutant prevention concentrations of isolates with various genetic constitutions prior to mutational analysis (122) and the stability of the selected resistance mechanisms (118) have been published.

a. Isolate Selection

Eleven previously characterized isolates were selected for single-step mutational studies based on QRDR substitutions and efflux phenotype. The genetic constitution, relevant to fluoroquinolone resistance, of the isolates and their ciprofloxacin, gatifloxacin, gemifloxacin, levofloxacin, and moxifloxacin MICs are listed in table 3.5.

Table 3.5 Ciprofloxacin, gatifloxacin, gemifloxacin, levofloxacin, and moxifloxacin MICs and the genetic constitution of the *S. pneumoniae* isolates used in the mutational studies

Isolate	Genetic constitution of isolates prior to mutational analysis			MICs (µg/mL)				
	ParC	GyrA	Efflux	Cipro	Gati	Gemi	Levo	Moxi
2670	-	-	Negative	1	0.25	0.016	0.5	0.06
2587	-	-	Negative	1	0.25	0.016	0.5	0.06
2663	-	-	Negative	1	0.125	0.008	0.5	0.06
15017	-	-	Positive	4	0.25	0.06	2	0.12
16072	-	-	Positive	4	0.5	0.06	2	0.12
4610	Ser79Phe	-	Negative	4	0.5	0.06	2	0.25
14744	Ser79Phe	-	Negative	4	0.5	0.06	2	0.25
1146	-	Ser81Phe	Negative	8	4	0.06	8	2
23786	-	Ser81Phe	Negative	4	4	0.12	8	1
984	Ser79Phe	Ser81Phe	Negative	8	4	0.12	8	2
17012	Ser79Phe	Ser81Phe	Negative	16	4	0.12	8	1

Cipro, Ciprofloxacin; Gati, Gatifloxacin; Gemi, Gemifloxacin; Levo, Levofloxacin; Moxi, Moxifloxacin; -, wild-type

b. Mutational Frequencies of Fluoroquinolone-resistant Laboratory Mutants Selected from Wild-Type (ParC and GyrA), Efflux-Positive or *S. pneumoniae* Isolates Containing GyrA and/or ParC Substitutions

The mutational frequencies observed for the fluoroquinolone-resistant mutants selected on ciprofloxacin, gatifloxacin, gemifloxacin, levofloxacin, and moxifloxacin from *S. pneumoniae* isolates that were wild-type (ParC and GyrA), efflux-positive or possessed GyrA and/or ParC substitutions are presented in table 3.6. The mutational frequencies presented are averages of the two or three isolates with each genetic background from which mutants were selected.

The mutational frequencies of the mutants selected on antibiotic plates at 1x, 2x, 4x, and 8x the MIC ranged between 10^{-1} and 10^{-4}, 10^{-1} and 10^{-8}, 10^{-2} and 10^{-9}, and 10^{-4} and 10^{-9}, respectively. The only mutants selected on antibiotic plates at 16x the MIC were selected at a frequency of 10^{-8} on moxifloxacin from isolates that had a ParC substitution prior to the mutational analysis.

The mutational frequencies for the mutants selected from the fluoroquinolone-susceptible isolates ranged between 10^{-1} and 10^{-8}. Similarly, the mutational frequencies for the mutants selected from the efflux-positive isolates ranged from 10^{-2} to 10^{-8}. For mutants selected from isolates with a ParC substitution, the mutational frequencies ranged between 10^{-3} and 10^{-8}. The mutational frequencies for the mutants selected from the isolates with a GyrA substitution ranged between 10^{-2} and 10^{-9}. For the mutants selected from isolates with substitutions in GyrA and ParC, the observed mutational frequencies ranged from 10^{-1} to 10^{-6}.

Table 3.6 **Mutational frequencies for single-step fluoroquinolone-resistant mutants selected on increasing concentrations of ciprofloxacin, gatifloxacin, gemifloxacin, levofloxacin, and moxifloxacin from *S. pneumoniae* isolates with various genetic backgrounds**

Selecting Fluoroquinolone	Selecting Concentration (fold of MIC)				
	1x	2x	4x	8x	16x
Wild-type GyrA & ParC, Efflux negative					
Ciprofloxacin	5.06E-03	2.56E-04	2.57E-05	9.50E-07	/
Gatifloxacin	4.60E-02	2.90E-05	1.80E-06	/	/
Gemifloxacin	4.13E-02	5.35E-03	1.11E-05	5.30E-07	/
Levofloxacin	2.80E-04	7.53E-06	/	/	/
Moxifloxacin	1.16E-01	9.80E-05	9.47E-07	3.50E-08	/
Wild-type GyrA & ParC, Efflux positive					
Ciprofloxacin	2.65E-04	2.52E-04	/	/	/
Gatifloxacin	2.86E-02	1.95E-04	1.70E-06	/	/
Gemifloxacin	4.40E-06	5.95E-08	/	/	/
Levofloxacin	2.50E-02	2.40E-04	/	/	/
Moxifloxacin	7.45E-02	1.46E-05	7.20E-07	/	/
ParC Ser79Phe, Wild-type GyrA, Efflux negative					
Ciprofloxacin	3.32E-04	7.09E-05	3.70E-07	1.80E-07	/
Gatifloxacin	4.40E-03	5.65E-07	1.46E-07	6.75E-08	/
Gemifloxacin	4.45E-06	4.90E-07	6.90E-08	1.00E-08	/
Levofloxacin	9.00E-06	1.55E-06	1.30E-07	5.65E-08	/
Moxifloxacin	9.51E-03	1.01E-06	1.92E-07	1.70E-07	1.50E-08
GyrA Ser81Phe, Wild-type ParC, Efflux negative					
Ciprofloxacin	1.10E-02	7.53E-05	4.65E-07	/	/
Gatifloxacin	2.20E-04	/	/	/	/
Gemifloxacin	6.89E-02	2.33E-05	1.06E-06	5.50E-08	/
Levofloxacin	1.34E-02	7.10E-06	/	/	/
Moxifloxacin	1.60E-02	5.50E-04	6.50E-09	6.50E-09	/
ParC Ser79Phe, GyrA Ser81Phe, Efflux negative					
Ciprofloxacin	3.70E-01	2.11E-01	2.00E-02	4.00E-04	/
Gatifloxacin	7.53E-03	8.50E-06	/	/	/
Gemifloxacin	4.90E-01	1.90E-01	1.90E-04	1.95E-06	/
Levofloxacin	2.10E-01	1.95E-04	/	/	/
Moxifloxacin	3.40E-01	9.01E-02	4.00E-05	/	/

/, no mutants selected at this fluoroquinolone concentration

c. Determination of the Mutant Prevention Concentrations of Wild-Type (ParC and GyrA), Efflux-Positive, or *S. pneumoniae* Isolates Containing GyrA and/or ParC Substitutions

Table 3.7 displays the mutant prevention concentrations of ciprofloxacin, gatifloxacin, gemifloxacin, levofloxacin, and moxifloxacin as both MPCs and MP_{MIC}s. The MPCs and MP_{MIC}s refer to the mutant prevention concentration in relation to the actual drug concentration and as a function of the fold MIC increase required to reach this level, respectively.

Table 3.7 Ciprofloxacin, gatifloxacin, gemifloxacin, levofloxacin, and moxifloxacin mutant prevention concentrations obtained for single-step fluoroquinolone-resistant mutants from *S. pneumoniae* isolates with various genetic backgrounds

Isolate	Ciprofloxacin		Gatifloxacin		Gemifloxacin		Levofloxacin		Moxifloxacin	
	MP_{MIC}	MPC	MP_{MIC}	MPC	MP_{MIC}	MPC	MP_{MIC}	MPC	MP_{MIC}	MPC
Wild-type GyrA & ParC, Efflux negative										
2587	16x	16	2x	0.5	8x	0.13	4x	2	8x	0.5
2663	8x	8	8x	1	16x	0.13	4x	2	8x	0.5
2670	8x	8	2x	0.5	4x	0.06	4x	2	16x	1
Wild-type GyrA & ParC, Efflux positive										
15017	4x	16	8x	2	4x	0.25	4x	8	8x	1
16072	4x	16	4x	2	4x	0.25	2x	4	4x	0.5
ParC Ser79Phe, Wild-type GyrA, Efflux negative										
4610	16x	64	16x	8	8x	0.5	16x	32	>16x	>4
14744	16x	64	16x	8	16x	1	16x	32	16x	4
GyrA Ser81Phe, Wild-type ParC, Efflux negative										
1146	8x	64	1x	4	8x	0.5	2x	16	2x	4
23786	8x	32	2x	8	16x	2	4x	32	4x	4
ParC Ser79Phe, GyrA Ser81Phe, Efflux negative										
984	16x	125	4x	16	16x	2	4x	32	4x	8
17012	8x	125	2x	8	16x	2	4x	32	8x	8

MPC, mutant prevention concentration (μg/mL); MP_{MIC}, mutant prevention concentration (fold increase of MIC)

For the mutants created from the fluoroquinolone-susceptible (wild-type ParC/GyrA and efflux-negative) isolates, the MPC order was ciprofloxacin >> levofloxacin > moxifloxacin = gatifloxacin > gemifloxacin (8-16 >> 2 > 0.5 - 1 = 0.5 - 1 > 0.06 - 0.13 μg/mL). The order of MP_{MIC}s was ciprofloxacin = moxifloxacin > gemifloxacin > levofloxacin > gatifloxacin (8 - 16x = 8 - 16x > 4 - 16x > 4x > 2 - 8x).

The mutants created from the efflux-positive isolates (wild-type ParC/GyrA) had an MPC order of ciprofloxacin > levofloxacin > gatifloxacin > moxifloxacin > gemifloxacin (16 > 4 - 8 > 2 > 0.5 - 1 > 0.25 μg/mL) and the MP_{MIC}s were 2 – 8x for all the fluoroquinolones.

The mutants created from the isolates with a ParC substitution (wild-type GyrA and efflux-negative), had a MPC order of ciprofloxacin > levofloxacin > gatifloxacin > moxifloxacin > gemifloxacin (64 > 32 > 8 > 4 > 0.5 - 1 μg/mL) and the MP_{MIC}s were 8 - >16x for all fluoroquinolones.

The MPC order for the mutants created from the isolates with a GyrA substitution (wild-type ParC and efflux-negative) was ciprofloxacin > levofloxacin > gatifloxacin > moxifloxacin > gemifloxacin (32 - 64 > 16 - 32 > 4 – 8 > 4 > 0.5 - 2 μg/mL) and the MP_{MIC} order was gemifloxacin > ciprofloxacin > levofloxacin = moxifloxacin > gatifloxacin (8 -16x > 8x > 2 – 4x = 2 – 4x > 1 - 2x).

The MPC order for the mutants created from the isolates with substitutions in GyrA and ParC (efflux-negative) was ciprofloxacin > levofloxacin > gatifloxacin > moxifloxacin > gemifloxacin (125 > 32 > 8 - 16 > 8 > 2 μg/mL) and the MP_{MIC} order was gemifloxacin > ciprofloxacin > moxifloxacin > levofloxacin > gatifloxacin (16x > 8 - 16x > 4 - 8x > 4x > 2 - 4x).

Based on the fluoroquinolones, the descending MPC order was generally isolates with substitutions in ParC and GyrA > isolates with a ParC substitution > isolates with a GyrA substitution > efflux-positive isolates > and fluoroquinolone-susceptible isolates. For levofloxacin, the MPCs were equivalent for isolates with substitutions in both ParC and GyrA and isolates with single ParC substitutions. The isolates with a ParC substitution and isolates with a GyrA substitution had equivalent MPCs for gemifloxacin and moxifloxacin. For moxifloxacin, the MPCs for the efflux-positive isolates were equal to those of the fluoroquinolone-susceptible isolates.

The order of MP_{MIC}s for ciprofloxacin was isolates with a ParC substitution > fluoroquinolone-susceptible isolates = isolates with substitutions in GyrA and ParC > isolates with a GyrA substitution > efflux-positive isolates.

For gatifloxacin, the order of MP_{MIC}s was isolates with a ParC substitution > efflux-positive isolates > fluoroquinolone-susceptible isolates = isolates with GyrA and ParC substitutions > isolates with a GyrA substitution.

The order of MP_{MIC}s for gemifloxacin was isolates with substitutions in GyrA and ParC > isolates with a single ParC or GyrA substitution = fluoroquinolone-susceptible isolates > and efflux-positive isolates.

For levofloxacin, the order of MP_{MIC}s was isolates with a ParC substitution > isolates with substitutions in GyrA and ParC = fluoroquinolone-susceptible isolates > efflux-positive isolates > isolates with a substitution in GyrA.

The order of MP_{MIC}s for moxifloxacin was isolates with a ParC substitution > fluoroquinolone-susceptible isolates > efflux-positive isolates = isolates with substitutions in GyrA and ParC > isolates with substitutions in GyrA.

d. Antimicrobial Susceptibility Testing by E-test

The fold increase between the isolates MICs, as determined by E-test, prior to mutational analysis and the most resistant mutant selected from each genetic background studied with each fluoroquinolone evaluated during the study are presented in table 3.8. The ciprofloxacin and levofloxacin MICs increased to the maximum MIC distinguishable by E-test, 32 μg/mL, in the mutants selected from isolates with a GyrA substitution and/or a ParC substitution. Gatifloxacin MICs of 32 μg/mL were observed for mutants selected with gatifloxacin, gemifloxacin or moxifloxacin from isolates with a ParC substitution, as well as mutants selected with gatifloxacin from isolates with GyrA and ParC substitutions, and mutants selected with gemifloxacin from isolates with a ParC substitution. Mutants selected with moxifloxacin from isolates with a ParC substitution demonstrated moxifloxacin MIC increases to 32 μg/mL.

For mutants selected from the fluoroquinolone-susceptible isolates, the greatest MIC increase was observed for ciprofloxacin followed by gemifloxacin, levofloxacin, gatifloxacin, and moxifloxacin.

The observed order of MIC increases for the mutants selected from efflux-positive isolates was greatest for ciprofloxacin, followed by moxifloxacin, gatifloxacin, and levofloxacin.

For mutants selected from the isolates with ParC substitutions, the greatest MIC increase was observed for moxifloxacin followed by gatifloxacin, levofloxacin, and ciprofloxacin; however, ciprofloxacin and levofloxacin MICs reached the maximum distinguishable MICs.

Table 3.8 Fold increase of ciprofloxacin, gatifloxacin, gemifloxacin, levofloxacin, and moxifloxacin MICs observed in the most resistant mutant selected with ciprofloxacin, gatifloxacin, gemifloxacin, levofloxacin, and moxifloxacin from isolates with various genetic backgrounds: susceptible [wild-type (ParC and GyrA)], efflux-positive, or *S. pneumoniae* isolates containing GyrA and/or ParC substitutions

Mutants Selected on	Fluoroquinolone tested	Genetic constitution of isolates prior to mutational analysis				
		Susceptible	Efflux	ParC	GyrA	GyrA & ParC
		MIC fold increase in most resistant mutant				
Cipro	**Cipro**	64*	4	7*	3*	1*
	Gati	6	2	54	3	4
	Gemi	8	ND	ND	ND	ND
	Levo	6	2	19*	1*	1*
	Moxi	3	1	37	4	1
Gati	**Cipro**	4	2	7*	3*	1*
	Gati	4	3	74*	4	5*
	Gemi	6	ND	ND	ND	ND
	Levo	4	2	19*	1*	1*
	Moxi	2	3	48	4	1.3
Gemi	**Cipro**	17	2	11*	3*	1*
	Gati	3	2	64*	4	2
	Gemi	4	ND	ND	ND	ND
	Levo	2	1	21*	1*	1*
	Moxi	2	2	126	4	1
Levo	**Cipro**	20	4	7*	3*	1*
	Gati	5	1	48	11*	2
	Gemi	8	ND	ND	ND	ND
	Levo	4	1	19*	1*	1*
	Moxi	5	1	40	5	1
Moxi	**Cipro**	23	2	4*	3*	1*
	Gati	12	3	84*	1	2
	Gemi	6	ND	ND	ND	ND
	Levo	24	2	16*	1*	1*
	Moxi	17	4	128*	1	4

Cipro, ciprofloxacin; Gati, gatifloxacin; Gemi, gemifloxacin; Levo, levofloxacin; Moxi, moxifloxacin; *, Maximum possible MIC increase; ND, no data

The observed order of MIC increases for the mutants selected from isolates with GyrA substitutions and isolates with GyrA and ParC substitutions was greatest for gatifloxacin, followed by moxifloxacin, ciprofloxacin and levofloxacin. As with the mutants selected from the isolates with ParC substitutions, the MIC increases for ciprofloxacin and levofloxacin reached the maximum distinguishable MICs.

e. Resistance Mechanisms Selected in Single-step Fluoroquinolone-Resistant Mutants

The resistance mechanisms, QRDR substitutions and efflux, selected in the laboratory mutants are summarized in figure 3.14. For the mutants selected from the fluoroquinolone-susceptible (wild-type ParC/GyrA and efflux-negative) isolates, 5/39 (13%) of the mutants had substitutions in GyrA (selected with moxifloxacin and gatifloxacin) and 3/39 (8%) of isolates had substitutions in ParC (selected with gemifloxacin). 21/39 (54%) of the mutants were positive for reserpine-sensitive ciprofloxacin efflux.

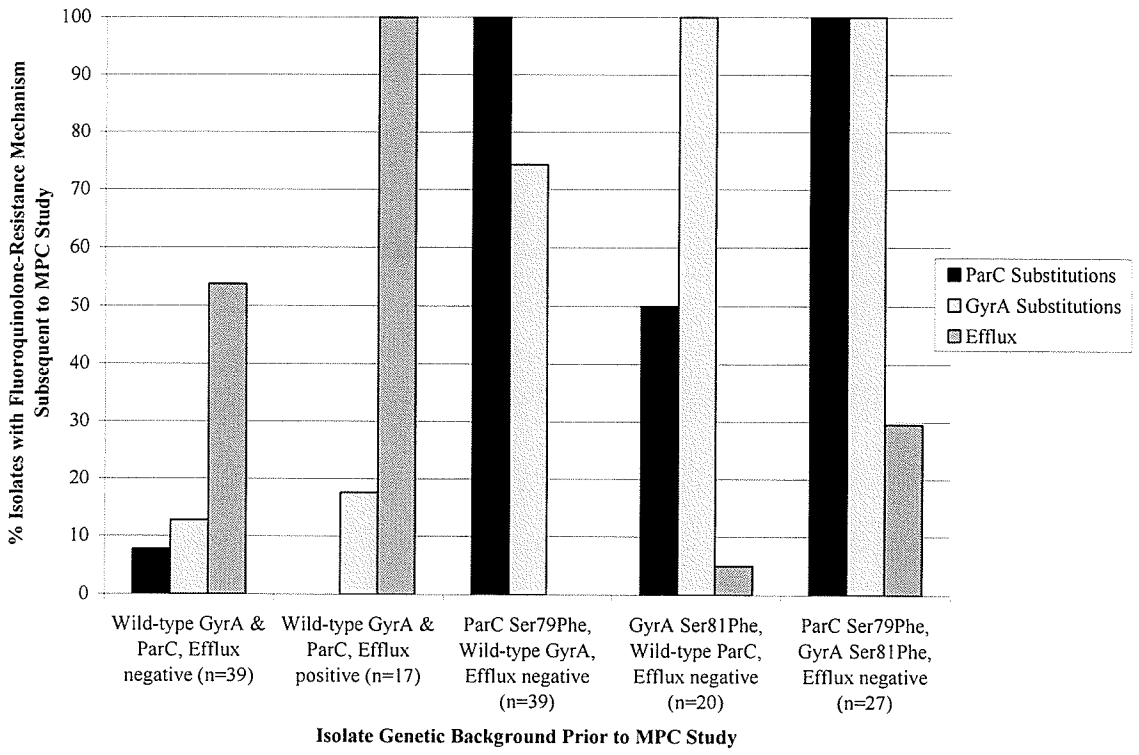
All the mutants selected from the efflux-positive isolates (wild-type GyrA and ParC) remained efflux positive. Only 3/17 (18%) also had QRDR substitutions. These changes occurred in GyrA and were selected with gatifloxacin and moxifloxacin.

29/39 (74%) of the mutants selected from the isolates with a ParC substitution (wild-type GyrA and efflux-negative) had secondary substitutions in GyrA and all were ciprofloxacin efflux-negative.

10/20 (50%) of the mutants selected from isolates with a GyrA substitution (wild-type ParC and efflux-negative) had secondary mutations in ParC and 1/20 (5%) was ciprofloxacin efflux-positive.

Figure 3.14 **Resistance mechanisms, QRDR substitutions and efflux, selected in single-step fluoroquinolone-resistant mutants based on original genotype: wild-type ParC/GyrA and efflux-negative isolates, wild-type ParC/GyrA and efflux-positive isolates, ParC: Ser79Phe substitution, wild-type GyrA and efflux-negative isolates, GyrA: Ser81Phe substitution, wild-type ParC and efflux-negative isolates, ParC: Ser79Phe, GyrA: Ser81Phe, and efflux-negative isolates. Resistant mutants were selected on ciprofloxacin, gatifloxacin, gemifloxacin, levofloxacin or moxifloxacin. Number of mutants selected in each genotype group indicated in parentheses.**

Fig. 3.14


% Isolates with Fluoroquinolone-Resistance Mechanism Subsequent to MPC Study
100
90
80
70
60
50
40
30
20
10
0
ParC Substitutions
GyrA Substitutions
Efflux
Wild-type GyrA & ParC, Efflux negative (n=39)
Wild-type GyrA & ParC, Efflux positive (n=17)
ParC Ser79Phe, Wild-type GyrA, Efflux negative (n=39)
GyrA Ser81Phe, Wild-type ParC, Efflux negative (n=20)
ParC Ser79Phe, GyrA Ser81Phe, Efflux negative (n=27)
Isolate Genetic Background Prior to MPC Study

None of the mutants selected from isolates with substitutions in GyrA and ParC had additional QRDR substitutions, but 8/27 (30%) were ciprofloxacin efflux-positive.

The specific QRDR substitutions selected in the single-step fluoroquinolone-resistant laboratory mutants are displayed in table 3.9. The most common substitution observed in GyrA was Ser81Tyr (86%). Other observed GyrA substitutions included Glu85Lys (8%) and Ser81Phe (6%). The most common substitution in ParC was Ser79Tyr (69%), followed by Asn91Asp (15%), Asn94Lys (8%), and Ser79Phe (8%).

f. Stability of Single-step Fluoroquinolone-Resistant Laboratory Mutants Compared to Clinical *S. pneumoniae* Isolates

The MIC decreases in the absence of antibiotic-selective pressure observed during the stability study are presented in table 3.10. MIC changes of greater than 2-fold were considered significant. The isolate 2663M4, an efflux-positive mutant that had been selected on moxifloxacin media, showed slight MIC decreases. During the course of study, the ciprofloxacin and moxifloxacin MICs decreased by 3.5 and 3-fold, respectively. Active efflux was still present at the termination of the study regardless of the observed MIC decreases. Each isolate had the same pulsed-field pattern prior to and following the stability study.

Table 3.9 **Specific resistance mechanisms, QRDR substitutions and efflux, selected in single-step fluoroquinolone-resistant laboratory mutants**

	Resistance mechanisms post mutational analysis (n with mechanism/total n in group)				
			Mutants selected on		
Isolate	**Ciprofloxacin**	**Gatifloxacin**	**Gemifloxacin**	**Levofloxacin**	**Moxifloxacin**
Wild-type GyrA & ParC, Efflux-negative					
2587	Efflux Pos. (4/4)	None observed	Efflux Pos. (2/3)	Efflux Pos. (2/2)	GyrA: Ser81Phe(1/3) Tyr (1/3), Efflux Pos. (1/3)
2663	ParC: Ser52Gy & Asn91Asp (1/3), Efflux Pos. (3/3)	None observed	ParC: Asn94Lys (1/4), Efflux Pos. (2/4)	ParC: Ser52Gy & Asn91Asp (1/2), Efflux Pos. (1/2)	Efflux Pos. (1/3)
2670	Efflux Pos. (3/3)	GyrA: Ser81Phe (1/1)	None observed	Efflux Pos. (1/2)	GyrA: Ser81Tyr (2/3)
ParC Ser79Phe, Wild-type GyrA, Efflux-negative					
4610	GyrA: Ser81Tyr (3/4)	GyrA: Ser81Tyr (3/3)	GyrA: Ser81Tyr (3/3)	GyrA: Ser81Tyr (3/4), Glu85Lys (1/4)	GyrA: Ser81Tyr (3/5), Glu85Lys (1/5)
14744	GyrA: Ser81Tyr (2/4)	GyrA: Ser81Tyr (2/4)	GyrA: Glu85Lys (1/4)	GyrA: Ser81Tyr (3/4)	GyrA: Ser81Tyr (3/4)
GyrA Ser81Phe, Wild-type ParC, Efflux-negative					
1146	ParC: Ser79Tyr (2/3)	/	ParC: Ser79Tyr (2/3)	ParC: Ser79Tyr (1/1)	ParC: Ser79Tyr (1/1)
23786	ParC: Ser79Tyr (1/3), Efflux Pos. (1/3)	ParC: Ser79Phe (1/1)	ParC: Ser79Tyr (1/4)	ParC: Ser79Tyr (1/2)	None observed
Wild-type GyrA & ParC, Efflux-positive					
15017	None observed	GyrA: Ser81Tyr (1/2)	None observed	None observed	GyrA: Ser81Tyr (2/2)
16072	None observed	None observed	None observed	None observed	None observed
ParC Ser79Phe, GyrA Ser81Phe, Efflux-negative					
984	Efflux Pos. (3/4)	Efflux Pos. (1/2)	Efflux Pos. (1/4)	Efflux Pos. (1/2)	Efflux Pos. (2/2)
17012	None observed	None observed	None observed	None observed	None observed

Neg., Negative; Pos., Positive; /, no mutant

Table 3.10 **Resistance mechanisms, QRDR substitutions in GyrA or ParC and efflux, present in the clinical and laboratory mutants evaluated for stability and observed MIC decreases over 20 days in the absence of antibiotic selective pressure.**

	Genetic constitution of isolates			MIC (μg/mL) fold decrease over the course of 20 days			
Isolate	**ParC substitution**	**GyrA substitution**	**Efflux**	**Cipro**	**Gati**	**Levo**	**Moxi**
Laboratory Mutants							
2663C4	None observed	None observed	Positive	0	0	0	0
2663Ga1	None observed	None observed	Positive	0	0	0	0
2663Ge8	None observed	None observed	Positive	0	0	1.5	0
2663L2	None observed	None observed	Positive	0	0	0	0
2663L1	None observed	Ser52Gly, Asn91Asp	Negative	0	0	0	0
2663M4	None observed	None observed	Positive	3.5	1.7	2	3
2663M2	None observed	None observed	Negative	0	0	0	0
15C2	None observed	None observed	Positive	0	0	1.3	0
15Ga4	None observed	Ser81Tyr	Positive	0	0	0	0
15Ge2	None observed	None observed	Positive	0	0	0	1.5
15L2	None observed	None observed	Positive	0	0	0	0
15M4	None observed	Ser81Tyr	Positive	0	0	0	0
15M2	None observed	Ser81Tyr	Positive	0	0	0	1.3
14C8	Ser79Phe	Ser81Tyr	Negative	0	0	0	0
14Ga8	Ser79Phe	Ser81Tyr	Negative	0	0	0	1.3
14Ge8	Ser79Phe	Glu85Lys	Negative	0	0	0	0
14L8	Ser79Phe	Ser81Tyr	Negative	0	0	0	0
14L4	Ser79Phe	Ser81Tyr	Negative	0	0	0	0
14M8	Ser79Phe	Ser81Tyr	Negative	0	0	0	0
14M4	Ser79Phe	Ser81Tyr	Negative	0	0	0	0
11C4	Ser79Tyr	Ser81Phe	Negative	0	0	0	0
11C2	Ser79Tyr	Ser81Phe	Negative	0	0	0	0
11Ge4	Ser79Tyr	Ser81Phe	Negative	0	0	0	1.3
11Ge2	Ser79Tyr	Ser81Phe	Negative	0	0	0	2
11L1	Ser79Tyr	Ser81Phe	Negative	0	0	0	0
11M1	Ser79Tyr	Ser81Phe	Negative	0	0	0	0
Clinical Isolates							
984	Ser79Phe	Ser81Phe	Negative	0	0	0	0
1146	None observed	Ser81Phe	Negative	0	0	0	0
1292	None observed	Ser81Tyr	Negative	0	0	0	1.3
2587	None observed	None observed	Negative	0	0	0	0
2663	None observed	None observed	Negative	0	0	0	0
2670	None observed	None observed	Negative	0	0	0	0
4610	Ser79Phe	None observed	Negative	0	0	0	0
10277	Ser79Phe	None observed	Positive	0	0	0	0

11361	Ser79Phe	Ser81Phe	Negative	0	0	0	0
12070	Ser79Tyr	None observed	Negative	0	0	0	0
12873	Ser79Phe	None observed	Positive	0	0	0	0
15017	None observed	None observed	Positive	0	0	0	0
16072	None observed	None observed	Positive	0	0	0	1.3
18705	Ser79Tyr	None observed	Positive	0	0	0	0
25074	Ser79Phe	None observed	Negative	0	0	0	0
27833	Ser79Tyr	Glu85Lys	Negative	0	0	0	1.3
28397	None observed	None observed	Positive	2	0	0	0

Cipro, Ciprofloxacin; Gati, Gatifloxacin; Levo, Levofloxacin, Gemi, Gemifloxacin

Part IV: Transformations of Fluoroquinolone Resistance-Associated Mutations into Susceptible *S. pneumoniae* Isolates

a. *S. pneumoniae* R6 Growth Curve

The colony counts at 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, and 12 hours and the OD_{600} values corresponding to those time points are displayed on figure 3.15. The key OD_{600} values for transformations with *S. pneumoniae* (0.04 and 0.2) are indicated on the figure.

b. Fluoroquinolone MICs of Transformants

Common ParC QRDR substitutions: Ser79Phe, Ser79Tyr, and Asp83Asn were transformed into fluoroquinolone-susceptible *S. pneumoniae* R6. All attempts to transform Ser79Phe and Lys137Asn together were unsuccessful. The recovered transformants had only the Ser79Phe substitution.

The fluoroquinolone MICs, tested by broth microdilution, prior to the transformations and subsequent to the transformations are presented in table 3.11. Prior to the transformations, the fluoroquinolone MICs of *S. pneumoniae* R6 were: ciprofloxacin 0.5 μg/mL, gatifloxacin 0.25 μg/mL, gemifloxacin 0.015 μg/mL, levofloxacin 0.5 μg/mL, and moxifloxacin 0.12 μg/mL. A Ser79Phe substitution in ParC resulted in MIC increases to: ciprofloxacin 4 μg/mL, gatifloxacin 0.5 μg/mL, gemifloxacin 0.06 μg/mL, levofloxacin 2 μg/mL, and moxifloxacin 0.25 μg/mL. A Ser79Tyr substitution in ParC resulted in MIC increases to: ciprofloxacin 4 μg/mL, gatifloxacin 0.5 μg/mL, gemifloxacin 0.06 μg/mL, levofloxacin 2 μg/mL, and moxifloxacin 0.5 μg/mL. An Asp83Asn substitution in ParC resulted in MIC increases to: ciprofloxacin 2 μg/mL, gatifloxacin 0.5 μg/mL, gemifloxacin 0.03 μg/mL, levofloxacin 2 μg/mL, and moxifloxacin 0.25 μg/mL. These results are confirmatory of

Figure 3.15 **Growth curve of *S. pneumoniae* R6 with colony counts and OD_{600} values over 12 hours. Cell densities of *S. pneumoniae* R6 grown in THYB over a 12 hour period with a starting inoculum of 4.5×10^6 CFU/mL were sampled at 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, and 12 hours. CFU/mL and the OD_{600} values were determine at each time point. The CFU/mL are plotted on a logarithmic scale. Key OD_{600} values for the transformations, 0.04 (■) and 0.2 (▲), are indicated on the growth curve.**

Fig. 3.15

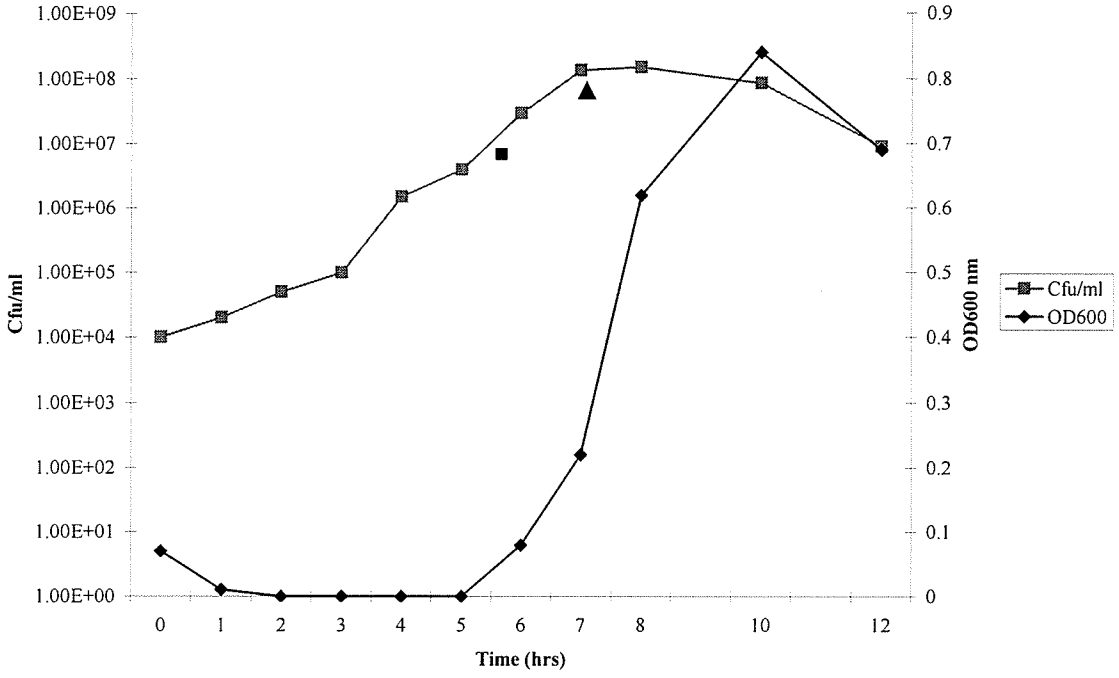

1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
Cfu/ml
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD600 nm
0 1 2 3 4 5 6 7 8 10 12
Time (hrs)
Cfu/ml
OD600

Table 3.11 Fluoroquinolone MICs of wild-type *S. pneumoniae* R6 and R6 subsequent to transformation of Ser79Phe, Ser79Tyr, and Asp83Asn.

Amino Acid Substitution	MICs (μg/ml)				
	Cipro	Gati	Gemi	Levo	Moxi
R6	0.5	0.25	0.015	0.5	0.12
Ser79Phe	4	0.5	0.06	2	0.25
Ser79Tyr	4	0.5	0.12	2	0.5
Asp83Asn	2	0.5	0.03	2	0.25

Cipro, ciprofloxacin; gati, gatifloxacin; gemi, gemifloxacin; levo, levofloxacin; moxi, moxifloxacin

those recently published by Korzheva *et al.* in which common ParC and GyrA substitutions were transformed into *S. pneumoniae* R6 (72). In general, the ciprofloxacin, gatifloxacin, gemifloxacin, levofloxacin, and moxifloxacin MICs reported here are one dilution higher than those reported by Korzheva *et al.* (72). Korzheva *et al.* described the MIC increases for ciprofloxacin, levofloxacin, gatifloxacin, moxifloxacin, norfloxacin, gemifloxacin, and garenoxacin in *S. pneumoniae* R6 transformed with common single ParC substitutions (Ser79Phe and Ser79Tyr), single GyrA substitutions (Ser81Phe and Ser81Tyr), and combinations of ParC and GyrA substitutions (Ser79Phe and Ser81Phe, Ser79Tyr and Ser81Tyr, Ser79Phe and Ser81Tyr, and Ser79Tyr and Ser81Phe).

D. DISCUSSION

Throughout this thesis, the hypothesis that the continued and increasing usage of fluoroquinolones in Canada will selectively genetically alter *S. pneumoniae* such that they will become increasingly fluoroquinolone resistant over time, but novel therapeutic approaches may limit this increase in resistance was investigated. Accordingly, three questions were studied. The first question was how have fluoroquinolone-resistant *S. pneumoniae* isolates evolved in Canada over 7 years? The second question was whether or not fluoroquinolone resistance-associated mutations are accumulating in fluoroquinolone-susceptible isolates, which have the potential to rapidly become highly resistant. Lastly, the question of whether the mutant prevention concentration theory, a novel dosing strategy, could limit the emergence of resistance was addressed. The answers to these questions and a discussion of how these results compare with current literature are presented in the following sections.

1. Molecular Characterization of Ciprofloxacin-resistant Canadian Clinical *S. pneumoniae* Isolates

a. Surveillance of Ciprofloxacin Resistance Over 7 Years

As the majority of respiratory infections likely caused by *S. pneumoniae* are treated empirically, surveillance of antimicrobial susceptibilities is essential in the determination of which antimicrobials are most likely to be effective. Surveillance is crucial to assess the current extent of resistance, characterize resistance patterns and mechanisms of resistance, and identify when new resistance patterns develop in the patient population (63). CROSS is one such surveillance study and it has significantly contributed to the knowledge of Canadian antimicrobial resistance development in

respiratory organisms during the past 7 years (136). Most importantly to the studies described in this thesis, CROSS has monitored fluoroquinolone (ciprofloxacin, gatifloxacin, gemifloxacin, levofloxacin, and moxifloxacin) resistance in *S. pneumoniae*. CROSS also monitors the resistance rates of penicillin, macrolides and many other antimicrobials.

S. pneumoniae infections are generally treated with β-lactams or macrolides; however, resistance to these antimicrobials has continued to increase in Canada and globally (135). In 2004, penicillin and clarithromycin resistance rates in Canadian *S. pneumoniae* isolates were 9.6% (25.3% non-susceptibility) and 13.4%, respectively (unpublished CROSS results). As the respiratory fluoroquinolones remain active against penicillin and macrolide-resistant *S. pneumoniae* (24), these agents are becoming increasingly used in the empiric therapy of *S. pneumoniae* infections. Fluoroquinolone resistance has remained low (<2%) for many years in Canada (27, 135, 137). However, the number of *S. pneumoniae* with reduced susceptibilities to the fluoroquinolones has been increasing recently mainly due to increased fluoroquinolone use (27). This highlights the need for continued surveillance of fluoroquinolone resistance in *S. pneumoniae*. By monitoring fluoroquinolone resistance, CROSS provides a means by which the hypothesis that fluoroquinolone resistance will increase over time in Canada can be addressed.

Throughout the last 7 years of CROSS, ciprofloxacin resistance increased significantly from 0.6% in 1997/98 to 3.7% in 2004 (p=0.0001). Ciprofloxacin resistance remained fairly stable in Canada between 1998/99 and 2003 with a dramatic increase from 2.4% in 2003 to 3.7% in 2004 (p=0.037). For levofloxacin, a gradual increase in

resistance was noted each year of CROSS from 0.2% in 1997/98 to 1.87% in 2004 (p=0.0001). Others groups also have reported significant increases in fluoroquinolone resistance in recent years. Doern *et al.* observed a substantial increase in fluoroquinolone resistance in the USA in 2001 that has since been maintained (38).

The resistance rate patterns of the other fluoroquinolones included in CROSS did not show as clear a trend as ciprofloxacin and levofloxacin. Gatifloxacin resistance fluctuated yearly between 1999/2000 and 2003 with a low in 2000/01 of 0.66% to a high of 0.97% in 2001/02. In 2004, an increase in gatifloxacin resistance to 1.56% was observed (2003 – 2004, p=0.097). Gemifloxacin resistance rates fluctuated yearly between 1999/2000 and 2004 with the lowest rate of 0.14% reported in 2001/02 and the highest rate of 0.7% in 2004. No consistent increase was observed as gemifloxacin resistance was higher in 1999/2000 and 2000/01 than in 2001/02 and 2003. Similarly, no consistent increases were observed in moxifloxacin resistance. Between 1997/98 and 2004, moxifloxacin resistance fluctuated between 0% and 0.47% with 6 of the 7 study years having resistance rates between 0.3 and 0.5%.

As fluoroquinolone resistance is associated with fluoroquinolone use (27, 106), the observed increase in fluoroquinolone resistance in 2004 may be associated with the total levels of fluoroquinolone use, the large amounts of ciprofloxacin used or the use of other less active fluoroquinolones, based on MICs, like levofloxacin. The use of older fluoroquinolones may select single *parC* mutations in *S. pneumoniae* isolates, which may then easily develop a secondary mutation and become resistant to most fluoroquinolones. These mutations may have been accumulating in fluoroquinolone-susceptible isolates. Accordingly, it is essential to determine the prevalence of resistance-associated

substitutions in fluoroquinolone-susceptible isolates. This question will be investigated at length in the second section of this discussion. The accumulation of these single *parC* mutations in previous years may have led to the significant increase in fluoroquinolone resistance observed in 2004.

Analyzing the data by age category, ciprofloxacin resistance significantly increased between 1997/98 and 2004 for adults (16-64 years, p=0.001) and elderly patients (>64 years, p=0.0001), but not for children, with the highest levels of resistance observed in the elderly. Other groups also have reported that the highest level of ciprofloxacin resistance occurs in elderly patients (27, 37, 106). The lack of resistance increase in children is expected as fluoroquinolones currently are not recommended for therapy in children so their exposure and potential for resistance selection are limited (82). If fluoroquinolones are approved for use in children, a significant rise in fluoroquinolone resistance will likely occur (82). There is a large amount of fluoroquinolone use in elderly patients, which provides an explanation for the observation that the highest levels of fluoroquinolone resistance occur in elderly patients (27).

The ciprofloxacin resistance rates for *S. pneumoniae* were also found to vary across the country with yearly fluctuations observed in each province. Regardless of the yearly fluctuations, all provinces had an increase in ciprofloxacin resistance over time. During the course of the study, average ciprofloxacin resistance rates were highest in Manitoba followed by Quebec, New Brunswick, Ontario, Alberta, British Columbia, Saskatchewan, Nova Scotia, and Prince Edward Island. In certain years, particular provinces had significantly higher resistance rates than the rest of the country. In 1998/99 and 2004, ciprofloxacin resistance rates in Quebec were significantly higher than

the rest of the country (p=0.034 and p=0.035). Similarly, in 2000/01, the Maritimes had higher ciprofloxacin resistance rates than the rest of Canada (p=0.005). In 2003, ciprofloxacin resistance in Manitoba was significantly higher than the rest of Canada (p=0.008). By PFGE and serotype results, these high provincial rates do not appear to be due to clonal outbreaks. In some instances, duplicate samples from patients with ciprofloxacin-resistant *S. pneumoniae* who visited a hospital on numerous occasions may have been included in CROSS. This may explain the significantly higher levels of ciprofloxacin resistance observed in particular provinces in certain years.

b. Molecular Characterization of the Ciprofloxacin-resistant *S. pneumoniae* in Canada

As antimicrobial resistance patterns evolve worldwide, the combination of genotypic and phenotypic surveillance is essential for the early detection of resistance mechanisms. The assessment of the frequency at which resistance mutations occur in a population has been identified as the "most refined means for tracking changes in fluoroquinolone resistance patterns" (38). Accordingly, all ciprofloxacin-resistant isolates collected as part of CROSS have been molecularly characterized. This study directly addresses the question of how fluoroquinolone-resistant *S. pneumoniae* isolates have evolved throughout the past 7 years.

i. Overview of Fluoroquinolone-Resistant *S. pneumoniae* Isolates

The majority of the 156 ciprofloxacin-resistant *S. pneumoniae* isolates collected between 1997/98 and 2004 as part of CROSS had substitutions in the QRDRs of GyrA and ParC (62%), followed by isolates with a ParC QRDR substitution (26%), isolates without QRDR substitutions in GyrA or ParC (10%), and isolates with a GyrA QRDR

substitution (2%). The ciprofloxacin resistance in 75% of the isolates with no QRDR substitutions in GyrA or ParC may be attributed to reserpine-inhibited efflux.

Between 1997/98 and 2004, there was a small decrease in the percent of ciprofloxacin-resistant isolates with either no QRDR substitutions or a single ParC substitution and an increase in the percent of isolates with substitutions in both GyrA and ParC. A non-significant decrease in ciprofloxacin-resistant isolates with single ParC substitutions has also been noted by Brueggemann *et al.* (23). The number of ciprofloxacin-resistant isolates with a single GyrA substitution (2%) remains too rare for analysis. Likewise, isolates with 3 QRDR substitutions in GyrA and ParC remain rare (3%). These isolates appeared in different years, were isolated from different provinces, had different substitutions, and belonged to different serotypes. These isolates likely occur randomly and have not been maintained in the population.

The percent of isolates with QRDR substitutions can be analyzed based on the ciprofloxacin, gatifloxacin, gemifloxacin, levofloxacin, and moxifloxacin MICs. As the ciprofloxacin MIC increased, the number of isolates with either no QRDR substitutions or single ParC substitutions decreased while the number of isolates with substitutions in GyrA and ParC increased. This trend was also observed with gatifloxacin, gemifloxacin, levofloxacin, and moxifloxacin. All isolates discussed thus far are ciprofloxacin-resistant; however, not all ciprofloxacin-resistant isolates are resistant to all fluoroquinolones. For this reason, the susceptible, intermediate, and resistant breakpoints for gatifloxacin, gemifloxacin, levofloxacin, and moxifloxacin are indicated on figures 3.6b – e. In each instance, numerous isolates considered susceptible or intermediate had single ParC substitutions, single GyrA substitutions or substitutions in both GyrA and

ParC. As these isolates had fluoroquinolone-susceptible MICs, a fluoroquinolone may be used in the treatment of these organisms. Isolates possessing single or double resistance-associated substitutions in GyrA and/or ParC are likely to result in clinical failure if treated with a fluoroquinolone (35). This highlights the inability of current susceptibility testing to identify isolates with fluoroquinolone resistance-associated substitutions (76, 80). These observations prompted the study of fluoroquinolone resistance-associated substitutions in fluoroquinolone susceptible isolates and the development of microbiological breakpoints to be discussed later.

ii. QRDR Substitutions in GyrA and ParC

The most frequently observed substitutions in ParC were at positions Ser79 (Ala, Phe or Tyr) (74% of ciprofloxacin-resistant isolates) and Asp83 (Ala, Asn, Gly or Tyr) (15% of ciprofloxacin-resistant isolates). The GyrA substitutions observed most often were at positions Ser81 (Phe or Tyr) (54% of ciprofloxacin-resistant isolates) and Glu85 (Gly or Lys) (10% of ciprofloxacin-resistant isolates). Overall, the most common genotype observed was Ser79Phe (ParC) and Ser81Phe (GyrA) (35% of ciprofloxacin-resistant isolates). The second most common genotype was isolates with a single ParC substitution (Ser79Phe) (13% of ciprofloxacin-resistant isolates). The high prevalence of these substitutions and their association with fluoroquinolone resistance are consistent with observations published by other investigators (11, 20, 23, 38, 66, 107). Substitutions at Ser79 in ParC and Ser81 in GyrA are believed to be the most commonly observed substitutions as these positions interact with the fluoroquinolone in the ternary complex (72). Much fluoroquinolone resistance research has focused on these substitutions as they are the most common substitutions associated with fluoroquinolone

resistance. Fluoroquinolone-resistant *S. pneumoniae* isolates with these GyrA and ParC substitutions have been shown to be inferior at colonization compared to isolates with wild-type genes (65). The low fluoroquinolone resistance rates currently reported may be partially explained by the decreased colonization ability of resistant isolates as colonization precedes infection (65). Although resistant isolates were less able to colonize, they were still able to cause lung infection; therefore, resistant isolates still cause severe disease and death (65). There is concern that these isolates could develop compensatory mutations enabling them to colonize as readily as wild-type isolates. Fluoroquinolone resistance could then rise dramatically.

While substitutions of Ser79 and Asp83 in ParC and Ser81 and Glu85 in GyrA were the most frequently observed amino acid alterations in this study, other substitutions were observed in ParC. Lys137Asn is commonly reported in surveillance studies, but it has not been associated with resistance (66, 72, 106). Lys137Asn was present in 25.6% of the ciprofloxacin-resistant isolates, but only in 2.6% of isolates with no other QRDR mutation. Our observations are even less than those reported by Richter *et al.* where 17% of isolates with single Lys137Asn substitutions were ciprofloxacin-resistant (72). Doern *et al.* reported an increase in the prevalence of substitutions such as Lys137Asn during recent years (38). A small increase, from 21% in 1998 to 30% in 2004 ($p=0.74$), in the prevalence of Lys137Asn has been observed in CROSS. Lys137Asn substitutions were associated with serotypes 23F and 9V in a study of fluoroquinolone resistance in penicillin-resistant clones in Spain (37). Similarly, the majority of isolates with Lys137Asn substitutions in CROSS were serotypes 9V, 12F, 14, and 23F. Thus, the

continued high prevalence of Lys137Asn substitutions may be less related to the fluoroquinolone resistance of the isolates and may be associated with their serotype.

Other substitutions were observed infrequently in this study, including Tyr59Asp, Asp78Ala, Ser107Tyr, and Glu120Gln in ParC. None of these substitutions were associated with MIC increases. Accordingly, they are unlikely to be involved in fluoroquinolone resistance. Asp78Ala/Asn substitutions have been reported previously, but as in this study, they usually occur with a substitution in GyrA so they either do not contribute to resistance or their effect is masked by the GyrA substitution (23, 38, 66).

iii. Efflux

Although the majority of ciprofloxacin resistance can be associated with QRDR substitutions in GyrA and ParC, fluoroquinolone resistance can also be due to efflux. Overall, 21.8% of the ciprofloxacin-resistant isolates were efflux-positive. The percent of efflux-positive isolates was highest among the low-level ciprofloxacin-resistant isolates (MICs 4 and 8 μg/mL). This observation was also reported by Bast *et al.* and they hypothesized that efflux may be down-regulated in high-level ciprofloxacin-resistant isolates (11). Until the fluoroquinolone efflux pump is identified in *S. pneumoniae*, the down-regulation of such a pump at high ciprofloxacin MICs is impossible to test.

Many have hypothesized that efflux permits cells to survive and replicate until a resistance-associated mutation develops (100, 128). In agreement with this concept, we observed that the highest percentage of efflux-positive isolates had no QRDR substitutions in GyrA or ParC (75%) whereas only 33% of isolates with a substitution in GyrA and 15% of isolates with either a single ParC substitution or substitutions in GyrA and ParC were also efflux-positive. As the majority of isolates without QRDR

substitutions in GyrA or ParC were observed in low-level ciprofloxacin-resistant isolates, it is logical that efflux is most common in low-level ciprofloxacin-resistant isolates.

Interestingly, the percent of efflux-positive ciprofloxacin-resistant *S. pneumoniae* isolates has decreased over the course of this study from 50% in 1997/98 to 19% in 2004 (p=0.19). The observed decrease in efflux may be explained by the observation that few isolates with substitutions in GyrA and ParC are efflux-positive and the number of these isolates has increased during the last 7 years.

iv. Substitutions in Full Genes of *gyrA*, *parC*, and *parE*

A large portion of the ciprofloxacin-resistant *S. pneumoniae* isolates without substitutions in GyrA or ParC may be resistant due to the presence of an efflux mechanism; however, there remain a number of isolates in this study whose resistance cannot be explained by QRDR substitutions or efflux and may have other factors contributing to their resistance. Some ciprofloxacin-resistant isolates do not have substitutions in the QRDRs of GyrA or ParC and are efflux-negative. Additionally, some isolates have a single QRDR substitution, but demonstrate high-level ciprofloxacin resistance. Previously, authors have hypothesized that mutations outside the QRDRs may play a role in fluoroquinolone resistance (24, 84, 95).

In order to address this hypothesis, the full genes of *gyrA*, *parC*, and *parE* were sequenced. Overall, 46 different amino acid substitutions were observed: 12 in GyrA, 19 in ParC, and 15 in ParE. Various non-QRDR substitutions were observed in GyrA, ParC, and ParE in both fluoroquinolone-susceptible and ciprofloxacin-resistant isolates (GyrA: Val489Ile and Ala653Thr, ParC: Ala450Val, Glu589Ala, Val608Ala, and Asp822Tyr, and ParE: Ile162Val). Two substitutions were observed only in fluoroquinolone-

susceptible isolates (ParC: Lys246Asn and ParE: Tyr263His). Throughout this study, 50% of isolates were found to have an Ile460Val substitution in ParE. This substitution is frequently reported in the literature; however, it has not been associated with increases in fluoroquinolone resistance (72). Substitutions solely occurring in ciprofloxacin-resistant isolates and appearing in 5% or more of isolates included Val486Ile and Ile711Val in GyrA, His373Arg, Ala394Thr, Lys473Asn, and Ala724Ser in ParC, and Ser132Asn and Thr216Ser in ParE. Of all substitutions observed, Ala191Val, Ser418Thr, Ala653Thr, Ile711Val, and Leu747Phe in GyrA, and Ala394Thr and Asp822Tyr in ParC, and Asp217Asn in ParE have been reported previously in ciprofloxacin-resistant *S. pneumoniae* (25).

Two general observations were made throughout this mutational analysis of the full genes of *gyrA*, *parC*, and *parE* in *S. pneumoniae*. Firstly, even though the greatest variety of non-QRDR substitutions was observed in ParC and the least in GyrA, non-QRDR substitutions were most common in GyrA (91%), followed by ParC (78%) and ParE (35%) in the ciprofloxacin-resistant isolates. This phenomenon was observed regardless of ciprofloxacin MIC. Secondly, non-QRDR substitutions may occur less frequently in isolates that already have a QRDR substitution in that particular gene. No particular substitution appeared to correlate with ciprofloxacin MIC or QRDR genotype.

Throughout this analysis, the resistance of some isolates was able to be explained, but the resistance of many isolates remained undetermined. One of the 3 ciprofloxacin-resistant isolates that had no GyrA or ParC QRDR substitutions and were efflux-negative had a known resistance substitution in ParE (Asp435Asn) (101), which accounts for its resistance. All 3 of the isolates with a single substitution in the QRDR of GyrA also had

the resistance-associated Asp435Asn substitution in ParE. The ciprofloxacin-resistant isolates with a single ParC QRDR substitution that had elevated ciprofloxacin MICs did not have any consistent non-QRDR substitutions to explain their increased MICs. Thus, it appears that non-QRDR substitutions are unable to account for the ciprofloxacin-resistant isolates with MICs greater than that expected due to their QRDR substitutions. By our analysis, the hypothesis that non-QRDR substitutions are associated with fluoroquinolone resistance is false. Other undetected resistance mechanisms must be present in these isolates. As of yet unidentified fluoroquinolone resistance-mediated efflux pumps may play a role. Alternatively, the isolates may have GyrB substitutions; however, the occurrence of such substitutions is rare and they are infrequently associated with fluoroquinolone resistance (106).

v. Serotypes and PFGE patterns

In addition to the identification of resistance-associated substitutions and reserpine-sensitive efflux in ciprofloxacin-resistant isolates, characterization was performed by serotyping and PFGE to determine if fluoroquinolone resistance is clonal in Canada. The 156 ciprofloxacin-resistant isolates belonged to 30 different serotypes. Although the ciprofloxacin-resistant isolates belonged to 30 serotypes, 10 serotypes accounted for 75% of all isolates. The most common serotypes were 6A (11 isolates), 6B (13 isolates), 9V (8 isolates), 11A (11 isolates), 12F (12 isolates), 14 (7 isolates), 19A (6 isolates), 19F (21 isolates), 22F (15 isolates), and 23F (13 isolates). These serotypes are frequently reported as the most common serotypes isolated from the nasopharynx (37, 55, 107). Reinert *et al.* found fluoroquinolone resistance to be associated with serogroup 19 with serotype 19A more prominent than 19F (106). This study also demonstrates that the

most common serogroup associated with fluoroquinolone resistance is 19, but 19F was observed more frequently than 19A.

The prevalence of serotypes was found to vary widely by year. Certain serotypes were observed only in one year of the study: 2, 3, 9A, 11D, 16F, 17F, 18B, 20, 31, 34, 35B, and a non-typeable isolate. These serotypes are also uncommon in other surveillance studies of ciprofloxacin-resistant isolates (107).

The 23-valent polysaccharide pneumococcal vaccines recommended for adults over 64 years of age contain serotypes: 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F. These vaccines provide coverage of 84.6% of the ciprofloxacin-resistant isolates identified in this study. 91% of the isolates recovered from patients over 64 years of age were covered by the 23-valent vaccines. Improved utilization of the 23-valent vaccines has the potential to severely limit infections caused by fluoroquinolone-resistant isolates as the majority of ciprofloxacin resistant isolates recovered throughout this study were from elderly patients and almost all of these isolates were covered by the vaccines. The only serotype commonly observed in this study that is not included in the 23-valent polysaccharide vaccines is 6A. Serotype 6A is a vaccine-related strain and cross-protection may be provided by the inclusion of serotype 6B in the 23-valent polysaccharide vaccine.

Correspondingly to the serotyping, PFGE revealed significant heterogeneity among the ciprofloxacin-resistant isolates. PFGE typing demonstrated that the majority of isolates investigated were genetically unrelated. Ninety-six (61.5%) of the ciprofloxacin-resistant isolates belonged to 14 different clusters of 4 or more organisms. This suggests that, for the most part, the ciprofloxacin-resistant isolates included in this

study arose independently, rather than through the clonal dissemination of a few highly resistant isolates.

Even within the clusters, there was considerable genetic heterogeneity with regards to fluoroquinolone resistance mechanisms and serotypes. Similar heterogeneity of the QRDR substitutions and serotypes within a PFGE cluster has been reported among fluoroquinolone-resistant isolates from the USA (107). For the CROSS isolates, each cluster included isolates with various QRDR substitutions in ParC and GyrA. None of the clusters consisted of isolates that all had identical ParC and GyrA QRDR substitutions. Similarly, different serotypes were observed within the clusters. Of the 14 clusters, only 4 consisted of isolates that all had the same serotype. Three clusters contained isolates that may have undergone capsular switching of 9V to 14 and 23F to 19F. These potential capsule switches have been previously identified (64, 106, 126).

Penicillin resistance has been associated with a limited number of serotypes. Recently, it has been shown that *S. pneumoniae* strains may acquire penicillin resistance and a new polysaccharide capsule from one donor strain as the penicillin binding proteins *pbp2x* and *pbp1a*, which are involved in penicillin resistance, are located near the capsular biosynthesis locus (126). This observation may explain the association of penicillin resistance with particular serotypes. The genes associated with fluoroquinolone resistance are distant from the capsular biosynthesis locus; thus, it is unlikely that fluoroquinolone resistance and a new polysaccharide capsule would be co-selected. Accordingly, a variety of serotypes were represented among the ciprofloxacin-resistant isolates. The diversity of QRDR substitutions and serotypes observed in the clusters highlights that although the isolates are genetically related; they have been

subjected to different antimicrobial exposures and have undergone new mutational events as opposed to clonal dissemination.

As part of the PFGE analysis, penicillin MICs were included for multi-drug resistance determination and to identify confounding factors had clonal dissemination been prevalent. Clonal dissemination of penicillin-resistant isolates has been demonstrated previously in Canada (90). Isolates were considered multi-drug resistant if they were resistant to penicillin and 2 other antimicrobials from different drug classes (107). In this study, multi-drug resistant isolates were resistant to ciprofloxacin and penicillin as well as clarithromycin, doxycycline or trimethoprim-sulfamethoxazole. Only 17 (10.9%) of the ciprofloxacin-resistant isolates were found to be multi-drug resistant. Four of the 17 multi-drug resistant isolates were in one cluster and 7 of the 17 were found in another cluster while the other 6 were scattered across the dendrogram. Nine (53%) of the multi-drug resistant isolates were isolated in the last 2 years of this study (2003 and 2004). The multi-drug resistant isolates were of serotypes 9V, 14, 19F, and 23F. These are the prominent serotypes reported internationally (37, 106, 107). Although the vast majority of isolates from this study are not multi-drug resistant, their apparent increase in the last 2 years and their association with the most prominent serotypes is of concern.

The recent increase in multi-drug resistant isolates is an indication that fluoroquinolone resistance is evolving in Canada. This supports our hypothesis that *S. pneumoniae* will be genetically altered over time in Canada as fluoroquinolone resistance increases. Some studies have noted fluoroquinolone resistance in multi-drug resistant international clones, which have the potential for rapid dissemination resulting in

significant increases in fluoroquinolone resistance (37, 106, 107). These multi-drug resistant clones are of concern as they can be selected by the use of many antimicrobials, not just fluoroquinolones (106). Thus, even with judicious use of the fluoroquinolones, if many of the multi-drug resistant international clones become fluoroquinolone-resistant, fluoroquinolone resistance could rise dramatically.

2. Fluoroquinolone Resistance-associated Substitutions in Canadian Clinical Fluoroquinolone-Susceptible *S. pneumoniae* Isolates

a. Fluoroquinolone Microbiological Breakpoints

As mentioned earlier in the discussion, numerous *S. pneumoniae* isolates considered susceptible or intermediate to gatifloxacin, gemifloxacin, levofloxacin, and moxifloxacin had single ParC substitutions, single GyrA substitutions, and substitutions in both GyrA and ParC. This highlights the inability of current susceptibility testing to identify isolates with fluoroquinolone resistance-associated substitutions (76, 80). These observations prompted the study of fluoroquinolone resistance-associated substitutions in fluoroquinolone-susceptible isolates for the determination of fluoroquinolone microbiological breakpoints. Highly resistant isolates with 2 mutations could be rapidly selected from isolates with undetected first-step mutations during fluoroquinolone treatment. Prior to theses studies, it was unknown if the prevalence of isolates with undetected first-step mutations was increasing in Canada or if microbiological breakpoints could be used to identify them.

The CLSI designs fluoroquinolone breakpoints utilizing various factors including frequency distributions, clinical data and pharmacokinetic/pharmacodynamic properties,

which incorporate the MIC, to determine the probability of bacteriological and clinical success, the detection of resistant populations or both (76, 86, 87). Breakpoints may be subdivided into clinical breakpoints and microbiologic breakpoints. Currently, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) defines clinical breakpoints and epidemiological cut-off values, which are MICs that separate wild-type and non-wild type organisms, whereas the CLSI does not (67). Rather, the CLSI focuses on clinical evidence as well as frequency distributions in setting clinical breakpoints. Clinical breakpoints are dependent on antimicrobial activity (MIC) as well as antimicrobial pharmacokinetics and pharmacodynamics. These breakpoints are derived in order to predict the probability of achieving bacteriological eradication from an infection site and ultimately achieving clinical success. On the other hand, microbiologic breakpoints are established to identify isolates that may be categorized as "susceptible" when applying clinical breakpoints but harbor resistance-associated mutations that have been associated with reduced susceptibility to that antimicrobial agent or antimicrobial class. Microbiologic breakpoints may thus be useful in monitoring the emergence of resistance, especially over time. Like the EUCAST epidemiology cut-off values, the microbiologic breakpoints separate wild-type organisms, isolates with no acquired or mutational resistance mechanisms to the particular antimicrobial, and non wild-type organisms, isolates with acquired or mutational resistance mechanisms for the evaluated antimicrobial (67).

Throughout this study, it became evident that the current CLSI breakpoints for fluoroquinolones and *S. pneumoniae* define many isolates as "susceptible" even though they harbor QRDR mutations. Based on the likelihood of QRDR mutations, we propose

microbiological resistance breakpoints for gatifloxacin, gemifloxacin, levofloxacin, and moxifloxacin. Our proposed microbiological resistance breakpoint is the MIC at which > 50% of the isolates carry QRDR substitutions. The proposed resistant microbiologic breakpoints are (μg/mL): gatifloxacin > 0.25, gemifloxacin > 0.03, levofloxacin > 1, and moxifloxacin > 0.12. These breakpoints would identify the majority of non-wild type isolates, which have the potential to develop high-level fluoroquinolone resistance subsequent to further exposure to fluoroquinolones.

The recent occurrence of treatment failures resulting from the use of levofloxacin in the treatment of community-acquired pneumonia caused by "susceptible" *S. pneumoniae* isolates that harbored QRDR substitutions (35, 48, 69) has led to the need to re-evaluate current breakpoints. It has previously been demonstrated that secondary mutations are acquired much more rapidly than first-step mutations resulting in highly resistant isolates (11), which have led to the observed treatment failures. As Lim *et al.* have recently suggested, emerging resistance patterns cannot be detected based on clinical breakpoints that are unable to identify first-step mutations (76). Thus, it is clinically important that we develop rapid identification methods for QRDR substitutions as to avoid treating a *S. pneumoniae* isolate carrying a first-step mutation with a fluoroquinolone in order to limit the development and propagation of highly resistant isolates.

Until such methodologies are developed, one mechanism to improve the chance of detecting an isolate with a resistance substitution is to consider the MICs of numerous fluoroquinolones. A much larger percentage of gatifloxacin, gemifloxacin, levofloxacin, and moxifloxacin-susceptible isolates were found to harbor resistance substitutions when

they were ciprofloxacin-resistant. Fluoroquinolone treatment should be avoided when an isolate is resistant to any of the fluoroquinolones, including ciprofloxacin. Other patient related factors can aid in the identification of isolates likely to have resistance substitutions such as treatment in a long-term care facility, hospitalization, and recent fluoroquinolone treatment, particularly in the last 3 months (48, 129).

b. Fluoroquinolone Resistance-associated Substitutions in Fluoroquinolone-Susceptible *S. pneumoniae* Isolates

Subsequently to the microbiological breakpoint study, a similar study was designed to increase the number of isolates evaluated and focus the investigation to ciprofloxacin-susceptible isolates with levofloxacin MICs of 1 μg/mL. This larger study permitted an analysis of the prevalence of fluoroquinolone-susceptible isolates with fluoroquinolone resistance-associated substitutions. Additionally, this study was designed to determine whether the prevalence of such isolates has increased over time. An accumulation of first-step mutations in *S. pneumoniae* may have contributed to the increasing fluoroquinolone resistance recently observed in Canada. Numerous groups have expressed concern that the use of respiratory fluoroquinolones, like levofloxacin, may be selecting a pool of fluoroquinolone-susceptible isolates with single ParC substitutions that may rapidly develop secondary substitutions in GyrA and become highly fluoroquinolone resistant (35, 36). Previous studies reported that between 59% and 71% of isolates with levofloxacin MICs of 2 μg/mL had QRDR substitutions in GyrA and/or ParC (36, 76). Few studies have evaluated isolates with levofloxacin MICs of 1 μg/mL. The MIC_{90} of levofloxacin for *S. pneumoniae* is 1 μg/mL (136). The use of

this MIC provides the greatest number of isolates for study and is a sensitive measure of shifts over time.

Isolates were selected from 1997 and earlier in order to test isolates that had not yet been exposed to the respiratory fluoroquinolones. Isolates from 2003 were also tested to identify changes in the prevalence of fluoroquinolone resistance substitutions in susceptible *S. pneumoniae* isolates subsequent to years of respiratory fluoroquinolone treatment. A non-significant increase, from 0.9% in pre-1998 isolates to 2.1% in 2003, in resistance-associated GyrA and ParC substitutions was observed (p=0.34).

These data do not support the hypothesis that the use of respiratory fluoroquinolones has resulted in an increase in the number of fluoroquinolone-susceptible isolates with substitutions in GyrA and/or ParC. However, an increasing trend was observed that may continue and become significant in the future. Another similar study will need to be conducted in a few years to ascertain if the increasing trend has continued and if so, if it has increased in a linear or exponential fashion. There is currently no way to determine what increases will be observed in the future, but it will clearly be important to the future of the fluoroquinolones.

This study further supports that the microbiological breakpoint for levofloxacin be set at a MIC of > 1 μg/mL. Isolates with MICs below this have very few resistance-associated substitutions whereas a considerable percent of isolates above the breakpoint have first-step substitutions. The use of such a breakpoint may reduce the probability of fluoroquinolone treatment failure due to unidentified first-step substitutions (76, 121). Various other factors may aid in the identification of situations likely to result in fluoroquinolone treatment failure such as the patient risk factors recently identified:

residence in a long-term care facility, hospitalization, and a history of fluoroquinolone use, particularly in the past 3 months (48, 129). Limiting the emergence of fluoroquinolone resistance and prolonging the utility of fluoroquinolones will be a multi-faceted approach.

3. Fluoroquinolone Mutant Prevention Concentrations in *S. pneumoniae*

a. Fluoroquinolone-resistant Mutants Selection

Dosing above the MPC has been suggested as a method by which the selection of mutants during antibiotic treatment could be minimized (41). The MPC also permits an evaluation of the differences in potencies of the fluoroquinolones (18, 41, 128). This study systematically examined the intrinsic development of fluoroquinolone resistance in *S. pneumoniae* by determining the mutational frequencies and MPCs of *S. pneumoniae* isolates with known genetic backgrounds exposed to ciprofloxacin, gatifloxacin, gemifloxacin, levofloxacin, and moxifloxacin. This study permitted an analysis of the hypothesis that novel therapeutic approaches, including the MPC as a dosing strategy, will limit the emergence of fluoroquinolone resistance.

Unlike other recent MPC studies of *S. pneumoniae* that rapidly screened hundreds of isolates with unknown genetic backgrounds (18), we chose to use a methodical, genetic approach. We carefully selected isolates that had known genetic backgrounds and characterized all isolates both prior to and following the MPC study.

Due to the time-consuming nature in which this study was conducted of genetically characterizing all isolates prior to and following the mutational analysis, only a few representative isolates could be evaluated. However, we felt it was vital to study the organism and the development of resistance in this protracted manner. In recent

studies by Blondeau *et al.* reporting on MPCs of fluoroquinolones for clinical isolates of *S. pneumoniae*, wide MPC ranges resulted as they tested a large number of isolates and did not genetically characterize their isolates prior to MPC testing (18). Our MPC range for each category of organisms, fluoroquinolone-susceptible (wild-type ParC/GyrA and efflux negative) isolates, isolates with a ParC substitution (wild-type GyrA and efflux negative), isolates with a GyrA substitution (wild-type ParC and efflux negative), isolates with substitutions in GyrA and ParC (efflux-negative), and efflux positive isolates (wild-type GyrA and ParC), was much smaller as the genetic constitution of the organisms was known prior to the study. The unsequenced clinical isolates in Blondeau's study may have contained ParC and/or GyrA amino acid substitutions that could account for their high MPC values.

This thorough mutational analysis of the respiratory fluoroquinolones has highlighted and revealed many important aspects of the future of fluoroquinolone therapy. Firstly, as the antibiotic concentrations in the selection media increased, the frequency at which mutants were selected decreased. No fluoroquinolone demonstrated consistently higher or lower mutational frequencies than any of the other fluoroquinolones studied.

Secondly, among the respiratory fluoroquinolones, gatifloxacin was the least likely, by MP_{MIC}, to select for resistance in ciprofloxacin-susceptible *S. pneumoniae* isolates followed by levofloxacin, gemifloxacin and moxifloxacin (in order). No differences existed between the fluoroquinolones in their likelihood of selecting mutants from efflux-positive isolates. Similarly, no difference was observed between the fluoroquinolones in their likelihood of selecting mutants from isolates with a ParC

substitution. Once a ParC substitution was present, the MPC increased dramatically for all fluoroquinolones. It has recently been noted in other MPC studies that *parC* mutations caused greater MPC increases than *gyrA* mutations (33, 34). Mutants selected from isolates with either a GyrA substitution or substitutions in GyrA and ParC were most likely, by MP_{MIC}, to be selected by gemifloxacin followed by ciprofloxacin, moxifloxacin, levofloxacin, and gatifloxacin (in order). An MPC study was recently conducted with levofloxacin and moxifloxacin in a rabbit model that also reported greater MPCs with levofloxacin than moxifloxacin with isolates that had either a single GyrA substitutions or substitutions in GyrA and ParC (33).

Sierra *et al.* recently correlated mutagenic potency of the fluoroquinolones to likelihood of mutant selection (116). They found levofloxacin and moxifloxacin to be less mutagenic than ciprofloxacin and gemifloxacin and resistant mutants to be selected most commonly by ciprofloxacin followed by gemifloxacin, moxifloxacin and levofloxacin (116). This order is similar to that observed for the mutants selected from fluoroquinolone-susceptible isolates, isolates with a single GyrA substitution or substitutions in GyrA and ParC but not for the mutants selected from efflux-positive isolates or isolates with a single ParC substitution.

The third major observation in this study was the gene in which mutations were selected corresponded to the known target preferences of the fluoroquinolones. Ciprofloxacin and levofloxacin selected primary substitutions in ParC. Gatifloxacin and moxifloxacin selected primary substitutions in GyrA. Although gemifloxacin has frequently been considered a dual-active fluoroquinolone, it selected primary substitutions in ParC. As has been reported previously, Ser to Tyr substitutions were

more common in the laboratory-selected mutants than Ser to Phe substitutions. The reverse is observed in clinical isolates. The transformation studies conducted as part of this project sought to determine if the resistance resulting from a substitution of Ser79 with a Phe differed from that of a Tyr. A similar resistance result regardless of the specific substitution would indicate that laboratory mutants can be used to study clinical isolates.

Fourthly, it was noted that mutants selected from efflux-positive isolates and fluoroquinolone-susceptible isolates behaved similarly in the selection of particular substitutions. Few substitutions were selected, if at all, in GyrA and approximately 15% of the mutants had substitutions in ParC. The presence or absence of an active efflux system does not appear to affect the selection of substitutions in GyrA or ParC. Substitutions were selected at low levels from isolates that did not have a primary substitution. Conversely, the majority of isolates with a ParC substitution readily acquired secondary substitutions in GyrA and became highly resistant to all fluoroquinolones. Similarly, half of the mutants selected from isolates with GyrA substitutions selected secondary substitutions and became highly resistant. The frequent selection of secondary substitutions and development of high-level fluoroquinolone resistance is disconcerting. This is particularly important in light of recent reports of the high percentage of isolates with levofloxacin MICs of 2 μg/mL (susceptible) that carry ParC substitutions (36, 76), as aforementioned. None of the mutants selected from isolates with substitutions in GyrA and ParC selected for additional QRDR substitutions, but 30% became efflux-positive. Isolates with three QRDR substitutions in GyrA and ParC were rarely observed in the clinical isolates and none were selected in the MPC

study. Two substitutions are sufficient to provide high-level fluoroquinolone resistance. It is possible that three substitutions in key positions in the enzymes carry too great a biological cost to be readily selected.

Lastly, although the selected substitutions corresponded to known target preferences, it was noted that the presence of a GyrA substitution or GyrA and ParC substitutions prior to MPC analysis had the greatest impact on the gemifloxacin and ciprofloxacin MP_{MIC}s. As gatifloxacin and moxifloxacin preferentially bind GyrA, we expected the largest MPC increase to be observed with those fluoroquinolones for mutants selected from isolates with GyrA substitutions. An explanation may be provided by recent work of Korzheva *et al.* who noted during transformation studies that substitutions in either ParC or GyrA resulted in similar MIC increases for levofloxacin and gatifloxacin. Although this differs from target specificity studies, they suggest that small differences in susceptibility may result in selective differences that are amplified in the mutant selection (72). The hypothesis, based on fluoroquinolone and *Staphylococcus aureus* studies, is interactions of a fluoroquinolone with either DNA gyrase or topoisomerase IV may result in cell death and cell sensitivities are determined directly by the most sensitive enzyme (72). However, with *S. pneumoniae*, susceptibilities to the fluoroquinolones have not been found to be solely associated with the most sensitive target; the less sensitive enzyme also plays a role in fluoroquinolone susceptibility (72). Susceptibility is highest when both enzymatic targets are wild-type. These results emphasize that much has yet to be learned about the processes between binding of the fluoroquinolones and cell death.

The MPC study emphasizes that although fluoroquinolone resistance in *S. pneumoniae* remains low, the opportunity for increased resistance exists as the use of fluoroquinolones for the treatment of respiratory tract infections rises (58, 137). The potential for resistance development should thus be considered when specific fluoroquinolones are selected for treatment. An MPC-based dosing strategy may be well-suited for the prevention of selection and propagation of fluoroquinolone resistance-causing substitutions from wild-type organisms. Unfortunately, as the MPCs increased dramatically for isolates that had a primary substitution, MPC dosing likely will not be able to circumvent the development of high-level fluoroquinolone resistance. The hypothesis that the MPC may be a novel therapeutic strategy that will limit the emergence of fluoroquinolone resistance is not fully supported.

b. Stability of Resistance Mechanisms

The stability of fluoroquinolone resistance mechanisms, chromosomal mutations and efflux, was determined in clinical and laboratory-derived resistant mutants. Nearly all fluoroquinolone resistance mechanisms remained stable in both clinical isolates and laboratory-selected mutants in the absence of antibiotic selective pressure. These findings demonstrate that laboratory-derived mutants are a valuable tool in the analysis of fluoroquinolone resistance development.

Additionally, this study implies that fluoroquinolone resistance mechanisms in *S. pneumoniae* are not likely to wane even without the selective pressure of the antimicrobials. Fluoroquinolone-resistant *S. pneumoniae* do not appear to spontaneously return to wild-type in the absence of fluoroquinolone selective pressure. Thus, we must limit the original development of fluoroquinolone resistance mechanisms. Once

fluoroquinolone resistance mechanisms become predominate in nature, we will not be able to reclaim the utility of these antimicrobial agents.

4. Transformations

As part of the characterization of fluoroquinolone resistance development in both clinical isolates and laboratory mutants, it was noted that Ser to Phe substitutions are most common in clinical isolates and Ser to Tyr substitutions are most common in laboratory-selected mutants. This transformation study was conducted to determine if the resistance profiles resulting from a Ser to Phe or Ser to Tyr substitution were similar. This was necessary in order to confirm that laboratory mutants were a reasonable tool in the analysis of how ciprofloxacin-resistant clinical isolates may be genetically altered during fluoroquinolone exposure. The transformations of *S. pneumoniae* R6 with Ser79Phe and Ser79Tyr substitutions in ParC resulted in identical MIC increases with ciprofloxacin, gatifloxacin, and levofloxacin and very similar increases with gemifloxacin and moxifloxacin. These results indicate that the laboratory-derived mutants are valuable tools in the evaluation of clinical fluoroquinolone resistance development.

Additionally, the transformations were intended to analyze whether the commonly observed Lys137Asn substitution affects fluoroquinolone resistance. All attempts at transforming the Lys137Asn substitution into *S. pneumoniae* R6 were unsuccessful. The transformation fragment containing the Lys137Asn substitution also had a Ser79Phe substitution. Ser79Phe is known to result in fluoroquinolone resistance; therefore, it provided a mechanism by which to select the transformants. The lack of transformation

of Lys137Asn may be explained by the observation that PCR fragments are readily taken up by the cell and recombined into the chromosome when sufficient homology exists between the resident DNA and the incoming fragment, but "end exclusion" has been observed in which the end of the donor molecule is less likely to be acquired by the recipient (12). Although a transformation fragment could have been amplified with Lys137Asn at the centre to eliminate the problem of end exclusion, the Lys137Asn substitution is not likely to cause a fluoroquinolone MIC increase. Therefore, it would have been impossible to select the transformants.

The transformations were ceased subsequent to the completion of those discussed above as a very thorough series of transformations was published by Korzheva *et al.* (72). The results of our transformations matched very closely with those published and it was determined that our further planned transformations would only serve to be confirmatory. Korzheva *et al.* also analyzed the Ser81Phe and Ser81Tyr substitutions in GyrA and found no difference between the resultant MICs for ciprofloxacin, gatifloxacin, garenoxacin, gemifloxacin, levofloxacin, moxifloxacin, and norfloxacin (72). Double substitutions in ParC and GyrA were also transformed including Ser79Phe/Ser81Phe, Ser79Phe/Ser81Tyr, Ser79Tyr/Ser81Tyr, and Ser79Tyr/Ser81Phe. All double substitutions resulted in very similar MIC increases, which were 4 – 16x higher than the MIC increases observed with single substitutions, depending on the fluoroquinolone (72). As all of the common substitutions in GyrA and ParC appear to result in similar MIC increases and our results were simply confirmatory of those previously published, the transformation experiments were discontinued.

5. Conclusions

The data collected throughout the course of this study support the hypothesis that continued and increasing usage of fluoroquinolones in Canada will selectively genetically alter *S. pneumoniae* such that fluoroquinolone resistance will increase over time. Surveillance and molecular analysis of fluoroquinolone resistance in *S. pneumoniae* in Canada was conducted to assess the question of how isolates had evolved during the 7 years of study. A dramatic rise in fluoroquinolone resistance has occurred in recent years.

In conjunction with the increase in resistance, genetic alterations related to fluoroquinolone resistance in *S. pneumoniae* have been observed. A slight increase in the prevalence of isolates with resistance substitutions in both GyrA and ParC resulting in high-level resistance was observed. An increase was also observed in the prevalence of fluoroquinolone-resistant isolates that were multi-drug resistant in recent years. These observations paint a disconcerting picture of increasing fluoroquinolone resistance in Canada and the potential demise of a highly effective and essential antimicrobial class.

In order to further investigate the rise in fluoroquinolone resistance, one objective of this thesis was to determine if resistance-associated substitutions were accumulating in fluoroquinolone-susceptible isolates. Indeed, the percentage of fluoroquinolone-susceptible isolates with resistance-associated substitutions appears to be slowly increasing in Canada. These isolates carry primary substitutions but remain undetected by current susceptibility-based methodologies. Upon fluoroquinolone treatment, these isolates have the potential to quickly become highly fluoroquinolone-resistant by rapid selection of secondary substitutions. A patient infected with a susceptible organism

carrying a substitution may be treated with a fluoroquinolone and subsequently fail therapy. Such occurrences may have contributed to the observed increase in the prevalence of isolates with resistance substitutions in GyrA and ParC. Thus, the study of fluoroquinolone resistance mechanisms in fluoroquinolone-susceptible *S. pneumoniae* isolates provides some insight into the increase in high-level fluoroquinolone resistance in Canada.

The second component of the hypothesis states that novel therapeutic approaches may limit the increase of fluoroquinolone resistance. The MPC work completed during this study demonstrated that it is a novel dosing strategy that is unlikely to prevent resistance emergence; therefore, the hypothesis was not supported. It was demonstrated throughout this study that the MPCs vary widely based on the original genetic makeup of the *S. pneumoniae* isolate. The MPC required to prevent the selection of secondary substitutions was exceptionally high. MPC dosing may be effective at limiting the selection of fluoroquinolone resistance from a wild-type population, but will be unable to remedy the difficulties recently highlighted with *S. pneumoniae* isolates carrying primary substitutions. The isolates circulating in Canada were shown to be genetically diverse during the molecular characterization section of this thesis. Accordingly, it is unlikely that MPC dosing would limit the emergence of fluoroquinolone resistance if it were applied clinically.

Other recently conducted research suggests that novel therapeutic approaches will limit the emergence of fluoroquinolone resistance and supports our original hypothesis. The microbiologic resistance breakpoints described herein may aid in the identification of *S. pneumoniae* isolates possessing primary substitutions. It is also apparent that the MICs

of more than one fluoroquinolone must be considered when an *S. pneumoniae* isolate is designated as fluoroquinolone-susceptible and appropriate for fluoroquinolone therapy. Additionally, other patient-related factors such as treatment in a long-term care facility, hospitalization, and/or a history of fluoroquinolone use, particularly within 3 months, have recently been reported as contraindications to fluoroquinolone therapy as such patients are more likely to be infected with isolates possessing primary substitutions (48, 129). The consideration of factors such as those listed above will greatly limit the inappropriate use of fluoroquinolones in the treatment of *S. pneumoniae* isolates possessing a primary substitution thereby lessening the increase in fluoroquinolone resistance. It must also be emphasized that improved vaccine utilization has the potential to reduce the number of infections with fluoroquinolone resistant isolates. In summary, novel therapeutic approaches may limit the increase of fluoroquinolone resistance in Canada, but the MPC is not one such strategy.

Throughout the course of this study, it has become apparent that fluoroquinolone resistance is evolving. The hypothesis that continued and increasing usage of fluoroquinolones in Canada will selectively genetically alter *S. pneumoniae* such that they will become increasingly fluoroquinolone resistant over time was supported by the research described herein. The second component of the hypothesis that novel therapeutic approaches may limit the increase in fluoroquinolone resistance is supported by the microbiological breakpoint study described throughout the thesis and patient-related factors described by other investigators, but not by the MPC conclusions.

It is clear that without rapid and appropriate intervention, high-level fluoroquinolone resistance in *S. pneumoniae* will likely become increasingly prevalent in

Canada. Various therapeutic considerations discussed herein have the potential to limit the increase of fluoroquinolone resistance in Canada. With prudent use and adaptation in the designation of *S. pneumoniae* isolates appropriate for fluoroquinolone therapy, the fluoroquinolone antimicrobials should provide excellent therapy for years to come. The demise of the fluoroquinolones is not inevitable.

6. Future Directions

With each question answered in research, many more are revealed. The data reported in this thesis provide a platform on which numerous future studies can be based. Some important studies that need to be conducted subsequent to this study are as follows:

1. The most apparent study requiring follow-up is the continuation of molecular surveillance of fluoroquinolone resistance in *S. pneumoniae* in Canada. It is essential that the observed increases in the prevalence of *S. pneumoniae* isolates with substitutions in GyrA and ParC and the association of fluoroquinolone resistance with multi-drug resistant isolates be carefully monitored. Additionally, it is vital to continue evaluating the fluoroquinolone-resistant *S. pneumoniae* isolates for clonality. The maintenance of such surveillance is valuable in the preparation of future therapy guidelines.
2. In order to accurately analyze the increasing trend of fluoroquinolone resistance-associated substitutions in fluoroquinolone-susceptible *S. pneumoniae* isolates, the study of susceptible isolates described herein will need to be repeated in a few years. The percent of isolates with

substitutions increased two-fold between 1997 and 2003, but the increase was considered non-significant. Another time point in this study would permit a true analysis of whether the increase will be linear and gradual or exponential.

3. The role of efflux in fluoroquinolone-resistant *S. pneumoniae* isolates has yet to be fully determined. As PmrA does not appear to be the only fluoroquinolone efflux pump in *S. pneumoniae* and it has not been shown to efflux the respiratory fluoroquinolones, the identity and role of other efflux pumps need to be elucidated. Only after the active pumps are identified will the role of efflux in fluoroquinolone resistance be able to be completely characterized.

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APPENDIX A

Demographics of the 156 ciprofloxacin-resistant *S. pneumoniae* isolates collected across Canada between 1997/8 and 2004

Isolate	Year	Centre	Location	Age	Sex
1282	97-98	Calgary Laboratory Services	Calgary, AB	60	F
4030	97-98	Calgary Laboratory Services	Calgary, AB	81	M
4610	97-98	Maisonneuve-Rosemont	Montreal, QC	77	M
9989	97-98	Novabyss Inc.	Sherbrooke, QC	77	M
10277	98-99	Montreal General Hospital	Montreal, QC	43	F
10280	98-99	Montreal General Hospital	Montreal, QC	69	F
10733	98-99	Victoria General Hospital	Victoria, BC	78	F
11361	98-99	St. Joseph's Hospital	Hamilton, ON	76	M
11434	98-99	Hotel-Dieu de Montreal	Montreal, QC	60	M
12291	98-99	Maisonneuve-Rosemont	Montreal, QC	70	M
12818	98-99	Calgary Laboratory Services	Calgary, AB	60	F
12873	98-99	South East Health Care Corporation	Moncton, NB	84	M
12883	98-99	South East Health Care Corporation	Moncton, NB	71	M
14033	98-99	St. Joseph's Hospital	Hamilton, ON	54	F
14744	98-99	Maisonneuve-Rosemont	Montreal, QC	76	M
14769	98-99	Maisonneuve-Rosemont	Montreal, QC	72	M
14904	98-99	Regina General Hospital	Regina, SK	67	F
15017	98-99	St. Joseph's Hospital	Hamilton, ON	62	F
16071	99-00	St. Boniface General Hospital	Winnipeg, MB	57	M
16072	99-00	St. Boniface General Hospital	Winnipeg, MB	64	M
16078	99-00	St. Boniface General Hospital	Winnipeg, MB	69	M
17012	99-00	Victoria General Hospital	Victoria, BC	91	F
17913	99-00	St. Joseph's Hospital	Hamilton, ON	70	F
18397	99-00	Mount Sinai Hospital	Toronto, ON	61	M
18410	99-00	Mount Sinai Hospital	Toronto, ON	90	F
18955	99-00	Queen Elizabeth II Health Sciences Centre	Halifax, NS	66	M
19103	99-00	Vancouver Hospital	Vancouver, BC	68	M
19120	99-00	Vancouver Hospital	Vancouver, BC	80	M
20336	99-00	Regina General Hospital	Regina, SK	70	M
20709	99-00	Victoria General Hospital	Victoria, BC	83	F
21181	99-00	South East Health Care Corporation	Moncton, NB	76	F
21288	99-00	Jewish General Hospital	Montreal, QC	94	F
22350	99-00	Regina General Hospital	Regina, SK	69	M
22360	99-00	Regina General Hospital	Regina, SK	70	M
22366	99-00	Regina General Hospital	Regina, SK	69	M

22627	99-00	Maisonneuve-Rosemont	Montreal, QC	62	M
22668	99-00	St. Joseph's Hospital	Hamilton, ON	79	M
22901	99-00	Montreal General Hospital	Montreal, QC	ND	ND
23070	00-01	Novabyss Inc.	Sherbrooke, QC	74	M
23335	99-00	University of Alberta Hospitals	Edmonton, AB	75	F
23448	00-01	London Health Sciences Centre	London, ON	67	M
23574	99-00	Health Sciences Centre	Winnipeg, MB	49	M
23786	00-01	South East Health Care Corporation	Moncton, NB	69	M
24086	00-01	Montreal General Hospital	Montreal, QC	35	F
25074	00-01	University of Alberta Hospitals	Edmonton, AB	48	F
25268	00-01	St. Joseph's Hospital	Hamilton, ON	50	F
26608	00-01	Royal University Hospital	Saskatoon, SK	76	M
27224	00-01	Novabyss Inc.	Sherbrooke, QC	85	F
27396	00-01	University of Alberta Hospitals	Edmonton, AB	47	M
27546	00-01	St. Joseph's Hospital	Hamilton, ON	86	F
27833	00-01	Maisonneuve-Rosemont	Montreal, QC	ND	F
27908	00-01	St. John Regional Hospital	St. John, NB	74	M
27917	00-01	St. John Regional Hospital	St. John, NB	80	M
28374	00-01	Mount Sinai Hospital	Toronto, ON	ND	F
28669	00-01	Vancouver Hospital	Vancouver, BC	61	M
29012	00-01	South East Health Care Corporation	Moncton, NB	60	M
29111	00-01	Maisonneuve-Rosemont	Montreal, QC	67	F
29228	00-01	Victoria General Hospital	Victoria, BC	73	M
29262	00-01	Queen Elizabeth II Health Sciences Centre	Halifax, NS	48	F
29265	00-01	Queen Elizabeth II Health Sciences Centre	Halifax, NS	57	F
29516	00-01	St. Joseph's Hospital	Hamilton, ON	51	F
29927	01-02	Montreal General Hospital	Montreal, QC	71	M
30890	01-02	St. Joseph's Hospital	Hamilton, ON	64	F
30900	01-02	St. Joseph's Hospital	Hamilton, ON	73	M
31685	01-02	St. Boniface General Hospital	Winnipeg, MB	91	M
32534	01-02	Maisonneuve-Rosemont	Montreal, QC	59	M
32549	01-02	Maisonneuve-Rosemont	Montreal, QC	58	F
32839	01-02	Health Sciences Centre	Winnipeg, MB	89	M
32867	01-02	Health Sciences Centre	Winnipeg, MB	37	F
33035	01-02	Regina General Hospital	Regina, SK	61	F
33726	01-02	Calgary Laboratory Services	Calgary, AB	80	F
33809	01-02	St. Joseph's Hospital	Hamilton, ON	74	F
34547	01-02	University of Alberta Hospitals	Edmonton, AB	56	M
34549	01-02	University of Alberta Hospitals	Edmonton, AB	56	M
34572	01-02	University of Alberta Hospitals	Edmonton, AB	92	M
34604	01-02	University of Alberta Hospitals	Edmonton, AB	80	M

35097	01-02	Mount Sinai Hospital	Toronto, ON	64	M
35152	01-02	Mount Sinai Hospital	Toronto, ON	80	M
39710	01-02	St. Boniface General Hospital	Winnipeg, MB	73	F
40810	01-02	St. Joseph's Hospital	Hamilton, ON	68	M
42372	01-02	Maisonneuve-Rosemont	Montreal, QC	76	M
44171	01-02	Vancouver Hospital	Vancouver, BC	72	F
45089	2003	Health Sciences Centre	Winnipeg, MB	40	F
45333	2003	St. Joseph's Hospital	Hamilton, ON	78	M
45336	2003	St. Joseph's Hospital	Hamilton, ON	84	M
45693	2003	St. Boniface General Hospital	Winnipeg, MB	37	F
45776	2003	Vancouver Hospital	Vancouver, BC	45	M
45780	2003	Vancouver Hospital	Vancouver, BC	21	F
46970	2003	University of Alberta Hospitals	Edmonton, AB	67	M
47209	2003	Jewish General Hospital	Montreal, QC	93	M
47224	2003	Jewish General Hospital	Montreal, QC	55	F
47225	2003	Jewish General Hospital	Montreal, QC	93	M
47396	2003	St. Boniface General Hospital	Winnipeg, MB	83	F
47789	2003	Maisonneuve-Rosemont	Montreal, QC	59	M
47797	2003	Maisonneuve-Rosemont	Montreal, QC	86	M
48198	2003	Health Sciences Centre	Winnipeg, MB	66	M
48486	2003	Queen Elizabeth II Health Sciences Centre	Halifax, NS	61	M
48865	2003	Health Sciences Centre	Winnipeg, MB	66	M
48866	2003	Health Sciences Centre	Winnipeg, MB	8	F
49101	2003	Calgary Laboratory Services	Calgary, AB	81	F
49322	2003	St. Boniface General Hospital	Winnipeg, MB	66	M
49710	2003	Mount Sinai Hospital	Toronto, ON	65	M
49711	2003	Mount Sinai Hospital	Toronto, ON	37	F
49755	2003	Mount Sinai Hospital	Toronto, ON	73	M
49773	2003	Mount Sinai Hospital	Toronto, ON	66	M
49928	2003	Health Sciences Centre	Winnipeg, MB	66	M
50154	2003	University of Alberta Hospitals	Edmonton, AB	26	F
50227	2003	Vancouver Hospital	Vancouver, BC	28	F
50418	2003	Hotel-Dieu de Montreal	Montreal, QC	71	M
50770	2003	St. John Regional Hospital	St. John, NB	81	M
50835	2003	Montreal General Hospital	Montreal, QC	74	F
50946	2003	Queen Elizabeth Hospital	Charlottetown, PEI	78	M
51126	2003	St. Boniface General Hospital	Winnipeg, MB	67	M
51531	2004	St. Joseph's Hospital	Hamilton, ON	53	F
51597	2004	Hamilton Heath Sciences Centre	Hamilton, ON	69	M
52418	2004	Montreal General Hospital	Montreal, QC	71	F
52651	2004	University of Alberta Hospitals	Edmonton, AB	79	M

52941	2004	Regina General Hospital	Regina, SK	90	M
53155	2004	Health Sciences Centre	Winnipeg, MB	19	M
53482	2004	St. Boniface General Hospital	Winnipeg, MB	55	F
53683	2004	Hamilton Heath Sciences Centre	Hamilton, ON	74	F
53908	2004	St. Joseph's Hosptial	Hamilton, ON	72	M
54610	2004	Health Sciences Centre	Winnipeg, MB	89	M
54883	2004	Novabyss Inc.	Sherbrooke, QC	68	M
55072	2004	Calgary Laboratory Services	Calgary, AB	51	M
55073	2004	Calgary Laboratory Services	Calgary, AB	66	M
55160	2004	Jewish General Hospital	Montreal, QC	74	M
55175	2004	Jewish General Hospital	Montreal, QC	77	F
55178	2004	Jewish General Hospital	Montreal, QC	85	M
55335	2004	Mount Sinai Hospital	Toronto, ON	80	M
55361	2004	Mount Sinai Hospital	Toronto, ON	57	M
55374	2004	Mount Sinai Hospital	Toronto, ON	85	F
55430	2004	St. Boniface General Hospital	Winnipeg, MB	73	M
55431	2004	St. Boniface General Hospital	Winnipeg, MB	56	M
55660	2004	St. Joseph's Hospital	Hamilton, ON	68	M
55663	2004	St. Joseph's Hospital	Hamilton, ON	80	M
55666	2004	St. Joseph's Hospital	Hamilton, ON	65	M
55719	2004	Regina General Hospital	Regina, SK	62	M
55798	2004	Ottawa Hospital	Ottawa, ON	34	M
56276	2004	St. Boniface General Hospital	Winnipeg, MB	64	M
56283	2004	St. Boniface General Hospital	Winnipeg, MB	67	M
56298	2004	Maisonneuve-Rosemont	Montreal, QC	70	M
56301	2004	Maisonneuve-Rosemont	Montreal, QC	87	M
56304	2004	Maisonneuve-Rosemont	Montreal, QC	74	M
56319	2004	Maisonneuve-Rosemont	Montreal, QC	76	M
56336	2004	Maisonneuve-Rosemont	Montreal, QC	77	F
56414	2004	Health Sciences Centre	Winnipeg, MB	57	M
56419	2004	Calgary Laboratory Services	Calgary, AB	78	M
56604	2004	St. Boniface General Hospital	Winnipeg, MB	64	M
56745	2004	Hotel-Dieu de Montreal	Montreal, QC	69	M
56765	2004	Hotel-Dieu de Montreal	Montreal, QC	69	F
56782	2004	Hotel-Dieu de Montreal	Montreal, QC	37	M
56904	2004	St. Boniface General Hospital	Winnipeg, MB	74	M
57155	2004	Victoria General Hospital	Vancouver, BC	56	M
57272	2004	Royal University Hospital	Saskatoon, SK	42	M
57278	2004	Royal University Hospital	Saskatoon, SK	72	F

AB, Alberta; BC, British Columbia; MB, Manitoba; NB, New Brunswick; NS, Nova Scotia; ON, Ontario; PEI, Prince Edward Island; QC, Quebec; SK, Saskatchewan; M, Male; F, Female; ND, No Data

APPENDIX B

Antibiogram of the 156 ciprofloxacin-resistant *S. pneumoniae* isolates collected across Canada between 1997/8 and 2004 (Pen, Clari and Cft MICs reproduced with permission from Dr. D. Hoban)

Isolate	MIC (µg/mL) of:							
	Cipro	Gati	Gemi	Levo	Moxi	Pen	Clari	Cft
1282	8	1	0.06	2	0.25	1	ND	0.5
4030	32	2	0.25	8	1	0.03	ND	0.06
4610	4	1	0.06	2	0.25	0.03	ND	0.06
9989	16	4	0.25	8	2	0.03	ND	0.06
10277	8	1	0.12	2	0.25	0.03	ND	0.06
10280	16	4	0.25	8	2	0.03	0.12	0.06
10733	16	8	1	16	4	0.03	1	0.06
11361	32	8	1	16	4	0.03	0.12	0.06
11434	4	0.5	0.06	1	0.12	1	0.25	0.5
12291	4	1	0.06	2	0.25	0.03	0.25	0.06
12818	8	2	0.25	8	1	0.03	ND	0.06
12873	8	1	0.12	2	0.25	0.06	0.12	0.06
12883	4	0.5	0.06	1	0.12	0.03	0.12	0.06
14033	32	8	1	32	4	0.03	0.12	0.06
14744	4	0.5	0.06	2	0.25	0.03	0.12	0.06
14769	8	0.25	0.03	2	0.25	1	ND	0.5
14904	8	4	0.5	8	4	2	0.5	1
15017	4	0.5	0.06	1	0.12	0.06	0.12	0.06
16071	16	4	0.06	8	2	0.03	0.03	0.06
16072	4	0.5	0.06	1	0.12	0.03	0.03	0.06
16078	32	8	0.5	16	2	0.03	0.03	0.06
17012	16	4	0.12	8	1	0.03	0.03	0.06
17913	8	0.5	0.06	2	0.25	0.03	0.03	0.06
18397	32	8	0.5	16	4	0.25	4	0.06
18410	16	4	0.25	8	2	0.03	0.03	0.06
18955	32	16	1	32	8	0.03	0.03	0.06
19103	4	0.25	ND	1	0.12	0.03	0.03	0.06
19120	16	1	0.12	2	0.25	0.03	128	0.06
20336	8	1	0.06	2	0.25	4	0.03	0.5
20709	16	4	0.25	8	2	0.03	0.03	0.06
21181	32	8	1	16	4	0.06	0.06	0.06
21288	16	8	2	16	8	1	2	1
22350	8	4	0.25	8	2	2	1	1
22360	8	1	0.12	2	0.25	1	0.06	0.5

22366	16	4	0.5	16	2	1	0.5	0.5
22627	8	0.5	0.06	2	0.25	0.03	0.03	0.06
22668	4	0.5	0.06	2	0.25	0.03	32	0.06
22901	16	2	0.25	4	1	0.06	0.06	0.06
23070	16	4	ND	16	2	0.06	0.03	0.06
23335	32	8	0.5	16	4	0.03	0.03	0.06
23448	4	1	0.12	2	0.25	0.03	4	0.06
23574	16	4	0.12	8	2	0.03	2	0.06
23786	4	4	0.12	8	1	0.06	0.03	0.06
24086	8	1	0.12	2	0.25	2	0.03	0.5
25074	4	0.5	0.06	2	0.25	0.5	0.03	0.06
25268	16	4	0.12	16	2	0.03	0.03	0.06
26608	16	4	0.25	8	2	0.06	0.03	0.06
27224	8	2	ND	2	2	0.03	0.03	0.06
27396	4	0.06	0.12	1	0.5	0.03	0.03	0.06
27546	32	8	0.5	16	4	0.06	0.03	0.06
27833	16	4	0.12	8	2	0.03	0.03	0.06
27908	4	0.5	0.06	2	0.25	4	0.03	2
27917	4	0.5	0.06	2	0.25	4	0.03	0.5
28374	32	8	0.5	16	2	0.06	0.03	0.12
28669	4	1	0.12	2	0.25	0.03	0.03	0.06
29012	4	0.5	0.03	1	0.25	8	1	0.5
29111	4	2	0.12	4	1	0.5	2	0.06
29228	16	4	0.25	16	2	0.12	0.03	0.06
29262	4	0.5	0.12	2	0.25	0.03	0.03	0.06
29265	16	8	0.25	16	4	0.03	0.03	0.06
29516	32	8	0.5	16	4	0.06	0.03	0.06
29927	16	4	0.25	16	2	0.03	0.03	0.06
30890	4	0.5	0.06	2	0.25	0.03	0.03	0.06
30900	4	4	0.06	2	2	0.06	0.03	0.06
31685	32	8	0.5	16	4	2	0.03	0.5
32534	16	4	0.25	16	4	0.03	0.03	0.06
32549	4	0.5	0.06	2	0.25	0.12	1	0.12
32839	8	4	0.12	8	2	0.03	0.03	0.06
32867	16	4	0.25	8	2	0.25	16	0.06
33035	32	4	0.5	16	2	0.03	0.03	0.06
33726	16	4	0.5	8	2	0.06	0.03	0.06
33809	16	2	0.25	32	2	0.03	0.03	0.06
34547	16	4	0.25	8	2	0.03	0.03	0.12
34549	16	8	0.5	8	2	0.03	0.03	0.06
34572	16	4	0.12	4	1	0.03	0.03	0.12
34604	4	0.5	0.06	2	0.25	0.03	0.03	0.06
35097	16	2	0.12	8	1	1	0.03	1

35152	4	0.5	0.06	2	0.25	0.06	4	0.06
39710	8	0.5	0.06	2	0.12	0.03	0.03	0.06
40810	16	4	0.5	8	2	0.06	0.03	0.06
42372	16	4	0.5	8	2	0.03	0.03	0.06
44171	4	1	0.06	2	0.25	0.06	0.03	0.06
45089	4	0.5	0.03	1	0.25	0.06	0.03	0.06
45333	16	4	0.25	8	1	4	1	1
45336	16	4	0.12	8	1	0.03	0.03	0.06
45693	16	2	0.25	8	2	0.03	0.03	0.06
45776	8	2	0.06	8	2	0.03	ND	0.06
45780	16	2	0.03	4	0.25	0.12	128	0.06
46970	16	4	0.25	8	2	0.06	0.03	0.06
47209	4	0.5	0.06	2	0.25	4	128	2
47224	4	0.5	0.06	2	0.25	4	8	4
47225	4	0.5	0.06	2	0.25	4	128	4
47396	32	8	0.5	16	2	0.12	0.03	0.06
47789	4	0.5	0.06	2	0.25	0.03	0.03	0.06
47797	32	16	0.25	32	8	0.03	0.03	0.06
48198	16	4	0.5	8	2	0.03	2	0.06
48486	32	8	0.5	16	2	0.03	0.03	0.06
48865	8	4	0.25	8	2	0.03	2	0.06
48866	16	8	0.5	16	4	0.03	2	0.06
49101	16	8	0.25	8	2	0.5	0.5	0.06
49322	4	2	0.12	4	1	0.03	0.5	0.06
49710	8	4	0.25	8	2	0.03	0.03	0.06
49711	8	2	0.5	8	2	0.03	0.03	0.06
49755	16	4	0.25	8	4	0.06	2	0.06
49773	16	4	0.25	16	4	0.06	1	0.06
49928	16	4	0.25	16	4	0.03	2	0.06
50154	4	0.5	0.06	2	0.25	0.12	0.03	0.06
50227	4	1	0.12	4	1	0.03	0.03	0.06
50418	4	2	0.12	4	1	4	128	1
50770	32	2	0.5	8	2	0.06	0.03	0.06
50835	4	2	0.12	4	1	0.03	0.03	0.06
50946	8	4	0.5	8	2	4	128	1
51126	4	2	0.12	4	1	0.03	8	0.06
51531	8	2	0.06	8	1	0.03	0.03	0.06
51597	4	0.25	0.03	2	0.06	0.03	0.03	0.06
52418	32	8	1	16	2	0.06	0.03	0.06
52651	8	2	0.25	8	2	0.03	0.03	0.06
52941	8	2	0.06	8	2	0.25	0.03	0.06
53155	8	4	0.25	8	4	0.06	2	0.06
53482	16	2	0.25	8	2	0.03	0.03	0.06

53683	4	4	0.12	2	1	0.03	0.03	0.06
53908	4	2	0.06	4	0.5	0.5	0.12	0.06
54610	4	1	0.06	2	0.25	0.03	0.03	0.06
54883	32	4	0.25	16	4	0.03	64	0.06
55072	4	0.5	0.06	2	0.25	0.03	2	0.06
55073	4	0.5	0.06	2	0.25	0.03	0.03	0.06
55160	8	4	0.25	8	2	0.03	0.03	0.06
55175	8	4	0.25	8	2	0.03	8	0.06
55178	8	4	0.25	8	2	0.03	4	0.06
55335	4	0.5	0.03	2	0.25	0.03	0.03	0.06
55361	4	0.5	0.03	2	0.12	0.06	0.03	0.06
55374	32	8	1	32	4	2	128	0.5
55430	16	2	0.25	8	2	0.12	1	0.12
55431	16	4	0.25	8	2	0.03	2	0.06
55660	8	4	0.25	8	2	0.03	0.03	0.06
55663	16	8	0.5	16	8	4	128	1
55666	8	4	0.12	4	2	0.03	0.03	0.06
55719	4	0.5	0.06	2	0.25	0.12	0.03	0.06
55798	4	0.5	0.12	2	0.12	0.03	0.5	0.06
56276	16	1	0.12	4	2	0.03	0.03	0.06
56283	8	4	0.25	8	2	0.03	2	0.06
56298	4	2	0.25	4	1	0.03	0.03	0.06
56301	4	0.5	0.06	2	0.25	1	128	0.25
56304	4	1	0.12	2	0.25	1	128	0.06
56319	4	0.5	0.06	2	0.25	0.03	0.03	0.06
56336	4	0.5	0.06	1	0.25	0.03	0.03	0.06
56414	4	0.5	0.06	2	0.25	0.06	0.03	0.06
56419	8	4	0.25	8	2	0.03	0.03	0.06
56604	8	4	0.5	8	2	0.03	0.03	0.06
56745	16	0.5	0.06	4	0.25	0.03	128	0.06
56765	4	0.5	0.06	2	0.25	0.06	0.03	0.06
56782	32	8	0.5	16	4	0.06	0.03	0.06
56904	32	8	0.5	16	4	1	128	0.12
57155	4	0.5	0.06	2	0.25	0.06	0.06	0.06
57272	32	4	0.25	16	2	0.25	0.5	0.06
57278	4	0.5	0.06	2	0.25	2	0.03	0.5

Cipro, Ciprofloxacin; Gati, Gatifloxacin; Gemi, Gemifloxacin; Levo, Levofloxacin; Moxi, Moxifloxacin; Pen, Penicillin; Clari, Clarithromycin; Cft, Cefotaxime; ND, No Data

APPENDIX C

Demographics, fluoroquinolone antibiogram, and substitutions in the QRDRs of GyrA and ParC of the 154 *S. pneumoniae* isolates studied for the determination of fluoroquinolone microbiological breakpoints

Isolate	Year	Location	MIC (µg/mL) of:					GyrA QRDR Substitutions	ParC QRDR Substitutions
			Cipro	Gati	Gemi	Levo	Moxi		
801	97-98	Victoria, BC	2	ND	ND	2	0.25	None observed	Asp83Asn
1282	97-98	Calgary, AB	4	0.5	0.06	2	0.25	None observed	None observed
3104	97-98	Winnipeg, MB	4	0.5	0.03	2	0.25	None observed	Ser79Phe
3447	97-98	Victoria, BC	1	ND	ND	0.5	0.06	None observed	None observed
3492	97-98	Regina, SK	1	ND	ND	1	0.12	None observed	None observed
3873	97-98	Halifax, NS	1	ND	ND	1	0.12	None observed	None observed
3979	97-98	Sherbrooke, QB	0.25	ND	ND	0.5	0.06	None observed	None observed
4455	97-98	Montreal, QB	2	ND	ND	2	0.25	None observed	None observed
4610	97-98	Montreal, QB	4	0.5	0.06	2	0.25	None observed	Ser79Phe
10158	98-99	London, ON	1	ND	ND	1	0.12	None observed	None observed
10250	98-99	Winnipeg, MB	2	ND	ND	2	0.25	None observed	None observed
10277	98-99	Montreal, QB	4	1	0.12	2	0.5	None observed	Ser79Phe
11059	98-99	Hamilton, ON	1	ND	ND	0.5	0.06	None observed	None observed
11434	98-99	Montreal, QB	4	0.5	0.03	2	0.06	None observed	Ser79Phe
11438	98-99	Montreal, QB	0.5	ND	ND	0.5	0.06	None observed	None observed
12070	98-99	Winnipeg, MB	4	1	0.06	2	0.25	None observed	Ser79Tyr
12208	98-99	Calgary, AB	2	ND	ND	2	0.25	None observed	None observed
12291	98-99	Montreal, QB	4	1	0.06	2	0.25	None observed	Ser79Tyr
12292	98-99	Montreal, QB	4	0.5	0.03	2	0.12	None observed	Ser79Phe
12547	98-99	Montreal, QB	4	0.5	0.03	2	0.12	None observed	Ser79Phe
12873	98-99	Moncton, NB	4	1	0.06	2	0.25	None observed	Ser79Phe
12883	98-99	Moncton, NB	4	0.5	0.03	2	0.25	None observed	Ser79Phe
14744	98-99	Montreal, QB	4	0.5	0.06	2	0.25	None observed	Ser79Phe
14769	98-99	Montreal, QB	8	0.25	0.03	2	0.25	None observed	Ser79Phe

15017	98-99	Hamilton, ON	4	0.25	0.06	2	0.12	None observed	None observed
16072	99-00	Winnipeg, MB	4	0.5	0.06	2	0.12	None observed	None observed
16539	99-00	Halifax, NS	1	0.25	0.015	1	0.12	None observed	None observed
17011	99-00	Victoria, BC	1	0.25	0.015	1	0.12	None observed	None observed
17484	99-00	Ottawa, ON	2	0.5	0.03	2	0.25	None observed	None observed
17723	99-00	Montreal, QB	2	0.25	0.03	1	0.12	None observed	None observed
17913	99-00	Hamilton, ON	8	0.5	0.06	2	0.25	None observed	Asp83Gly
18705	99-00	Hamilton, ON	4	0.5	0.06	2	0.25	None observed	Ser79Tyr
18720	99-00	Ottawa, ON	0.25	0.06	0.015	0.5	0.06	None observed	Asn91Asp
18922	99-00	Moncton, NB	1	0.25	0.03	1	0.12	None observed	None observed
19103	99-00	Vancouver, BC	4	0.25	ND	1	0.12	None observed	None observed
19120	99-00	Vancouver, BC	16	0.25	0.25	2	0.12	None observed	Asp83Asn
19519	99-00	Hamilton, ON	1	0.25	0.03	1	0.12	None observed	None observed
19839	99-00	Montreal, QB	0.25	0.06	0.015	0.5	0.06	None observed	None observed
19840	99-00	Montreal, QB	0.5	0.12	0.015	0.5	0.06	None observed	None observed
20336	99-00	Regina, SK	8	0.5	0.03	2	0.25	None observed	Asp83Ala
21473	99-00	Calgary, AB	0.25	0.12	0.015	0.5	0.06	None observed	None observed
22203	99-00	Vancouver, BC	4	0.5	0.03	2	0.25	None observed	None observed
22360	99-00	Regina, SK	8	0.25	0.03	2	0.25	None observed	Asp83Ala
22623	99-00	Montreal, QB	0.5	0.12	0.015	0.5	0.06	None observed	None observed
22627	99-00	Montreal, QB	4	0.25	0.015	2	0.25	None observed	Asp83Gly
22668	99-00	Hamilton, ON	4	0.5	0.03	2	0.25	None observed	Ser79Tyr
22784	99-00	Saskatoon, SK	2	0.5	0.015	2	0.25	None observed	None observed
23063	00-01	Winnipeg, MB	1	0.25	0.015	1	0.12	None observed	None observed
23448	00-01	London, ON	4	0.5	0.06	2	0.25	None observed	Ser79Phe
23493	00-01	Winnipeg, MB	1	0.25	0.015	1	0.12	None observed	None observed
23536	00-01	Winnipeg, MB	0.5	0.12	0.008	0.5	0.06	None observed	None observed
24086	00-01	Montreal, QB	8	1	0.03	2	0.5	None observed	Tyr59Asp
24091	00-01	Montreal, QB	1	0.12	0.015	0.5	0.06	None observed	None observed
24120	00-01	Winnipeg, MB	2	0.5	0.06	2	0.25	None observed	Ser79Phe
25074	00-01	Edmonton, AB	4	0.5	0.03	2	0.25	None observed	Ser79Phe

25506	00-01	Winnipeg, MB	1	0.25	0.03	1	0.12	None observed	None observed
26393	00-01	Sherbrooke, QB	0.5	0.12	0.008	0.5	0.06	None observed	None observed
26608	00-01	Saskatoon, SK	16	4	0.12	2	0.5	Ser81Phe	Ser79Phe
27224	00-01	Sherbrooke, QB	8	2	ND	2	2	Ser81Tyr	Ser79Phe
27396	00-01	Edmonton, AB	4	0.5	0.12	1	0.5	None observed	Ser79Phe
27406	00-01	Edmonton, AB	1	0.25	0.03	1	0.12	None observed	None observed
27908	00-01	St. John, NB	4	0.5	0.03	2	0.25	None observed	Asp83Asn
27917	00-01	St. John, NB	4	0.5	0.03	2	0.25	None observed	Asp83Asn
27991	00-01	Calgary, AB	0.5	0.25	0.03	1	0.12	None observed	None observed
28102	00-01	Toronto, ON	0.25	0.12	0.008	0.25	0.06	None observed	None observed
28364	00-01	Toronto, ON	1	0.25	0.03	1	0.12	None observed	None observed
28368	00-01	Toronto, ON	4	0.5	0.015	2	0.25	None observed	None observed
28397	00-01	Toronto, ON	4	0.5	0.015	1	0.25	None observed	None observed
28669	00-01	Vancouver, BC	4	0.5	0.03	1	0.25	None observed	Ser107Tyr
29012	00-01	Moncton, NB	4	0.5	0.06	2	0.25	None observed	None observed
29098	00-01	Montreal, QB	2	0.5	0.03	2	0.25	None observed	Ser79Phe
29245	00-01	Halifax, NS	4	1	0.06	2	0.5	Glu85Lys	None observed
29248	00-01	Halifax, NS	2	0.5	0.03	2	0.25	None observed	None observed
29262	00-01	Halifax, NS	4	0.5	0.06	2	0.25	None observed	None observed
29317	00-01	Ottawa, ON	2	0.5	0.03	2	0.25	None observed	None observed
29377	00-01	London, ON	1	0.5	0.03	2	0.25	None observed	None observed
29403	00-01	Ottawa, ON	2	0.5	0.03	2	0.25	None observed	None observed
29453	00-01	Regina, SK	2	0.5	0.06	2	0.25	None observed	Leu30Phe, Tyr46Asp
29460	00-01	Regina, SK	2	0.5	0.06	2	0.25	None observed	None observed
29496	00-01	Hamilton, ON	1	0.5	0.03	2	0.25	None observed	None observed
29523	01-02	Winnipeg, MB	1	0.5	0.03	2	0.25	None observed	None observed
29644	01-02	Winnipeg, MB	2	0.5	0.03	2	0.25	None observed	None observed
29929	01-02	Montreal, QB	0.5	0.25	0.015	1	0.12	None observed	None observed
30115	01-02	Winnipeg, MB	2	0.5	0.03	2	0.25	Gly54Cys	None observed
30462	01-02	Saskatoon, SK	2	0.5	0.03	2	0.25	None observed	Ser79Phe

30478	01-02	Ottawa, ON	1	0.25	0.015	1	0.12	None observed	None observed
31003	01-02	Halifax, NS	0.5	0.12	0.008	0.5	0.06	None observed	None observed
31173	01-02	Charlottetown, PEI	0.5	0.12	0.008	0.5	0.06	None observed	Asp78Ala
31318	01-02	Ottawa, ON	2	0.25	0.03	1	0.12	None observed	None observed
31831	01-02	Ottawa, ON	0.5	0.12	0.008	0.5	0.06	None observed	None observed
32382	01-02	Regina, SK	0.5	0.12	0.008	0.5	0.06	None observed	None observed
32393	01-02	Regina, SK	0.5	0.12	0.008	0.5	0.06	None observed	None observed
32480	01-02	Montreal, QB	2	0.5	0.03	2	0.25	None observed	Glu135Asp
32541	01-02	Montreal, QB	0.5	0.12	0.008	0.5	0.06	None observed	None observed
34860	01-02	St. John, NB	2	0.5	0.03	2	0.25	None observed	None observed
35181	01-02	Toronto, ON	0.5	0.12	0.008	0.5	0.06	None observed	Ser52Gly
35599	01-02	Vancouver, BC	2	0.5	0.03	2	0.25	None observed	Ser52Gly, Asn91Asp
39727	01-02	London, ON	1	0.25	0.03	1	0.12	None observed	None observed
42745	01-02	Halifax, NS	1	0.25	0.03	1	0.12	Asp58Tyr	None observed
43780	01-02	Hamilton, ON	0.5	0.12	0.008	0.5	0.06	None observed	None observed
43805	01-02	Edmonton, ON	0.5	0.25	0.03	1	0.12	None observed	None observed
44443	2003	Winnipeg, MB	1	0.25	0.015	1	0.12	None observed	None observed
44889	2003	London, ON	2	0.5	0.03	1	0.12	None observed	None observed
45685	2003	Moncton, NB	0.5	1	0.015	1	0.5	None observed	None observed
45777	2003	Vancouver, BC	0.5	0.25	0.008	0.5	0.12	None observed	None observed
45783	2003	Vancouver, BC	1	0.5	0.03	1	0.25	None observed	None observed
45864	2003	Moncton, NB	0.5	1	0.015	1	0.5	None observed	None observed
45966	2003	London, ON	1	0.25	0.015	1	0.12	None observed	None observed
46039	2003	Hamilton, ON	1	0.25	0.015	1	0.25	None observed	None observed
46194	2003	St. John, NB	1	0.25	0.015	1	0.12	None observed	None observed
46196	2003	St. John, NB	1	0.25	0.015	1	0.12	None observed	None observed
46312	2003	Montreal, QC	1	0.25	0.015	1	0.12	None observed	None observed
46658	2003	Winnipeg, MB	1	0.25	0.015	1	0.12	None observed	None observed
46676	2003	Calgary, AB	1	0.25	0.008	1	0.12	None observed	None observed
46679	2003	Calgary, AB	0.5	0.25	0.015	1	0.12	None observed	None observed

46710	2003	Calgary, AB	1	0.25	0.008	1	0.12	None observed	None observed
46899	2003	Winnipeg, MB	1	0.25	0.015	1	0.12	None observed	None observed
47071	2003	Edmonton, AB	0.5	0.25	0.008	1	0.12	None observed	None observed
47077	2003	Edmonton, AB	1	0.25	0.008	1	0.12	None observed	None observed
47087	2003	Edmonton, AB	1	0.25	0.008	1	0.12	None observed	None observed
47092	2003	Sydney, NS	1	0.25	0.015	1	0.12	None observed	None observed
47130	2003	Ottawa, ON	1	0.25	0.015	1	0.12	None observed	None observed
47131	2003	Ottawa, ON	1	0.25	0.015	1	0.12	None observed	None observed
47137	2003	Ottawa, ON	1	0.25	0.015	1	0.12	None observed	None observed
47292	2003	Montreal, QC	0.5	0.25	0.015	1	0.12	None observed	None observed
47303	2003	Montreal, QC	1	0.25	0.015	1	0.12	None observed	None observed
47380	2003	Hamilton, ON	1	0.25	0.015	1	0.12	None observed	None observed
47528	2003	Halifax, NS	1	0.25	0.015	1	0.12	None observed	None observed
47531	2003	Halifax, NS	1	0.25	0.015	1	0.12	None observed	None observed
47532	2003	Halifax, NS	1	0.25	0.015	1	0.12	None observed	None observed
47533	2003	Halifax, NS	1	0.25	0.015	1	0.12	None observed	None observed
47760	2003	Hamilton, ON	1	0.25	0.03	1	0.12	None observed	None observed
47823	2003	Montreal, QC	1	0.25	0.03	1	0.12	None observed	None observed
47824	2003	Montreal, QC	1	0.25	0.015	1	0.12	None observed	None observed
47924	2003	Victoria, BC	1	0.25	0.015	1	0.12	None observed	None observed
47925	2003	Victoria, BC	1	0.25	0.015	1	0.12	None observed	None observed
47935	2003	Victoria, BC	1	0.25	0.015	1	0.12	None observed	None observed
48107	2003	Winnipeg, MB	1	0.25	0.015	1	0.12	None observed	None observed
48160	2003	Saskatoon, SK	1	0.25	0.03	1	0.12	None observed	None observed
48163	2003	Saskatoon, SK	1	0.25	0.03	1	0.12	None observed	None observed
48348	2003	Toronto, ON	1	0.5	0.015	1	0.12	None observed	None observed
48350	2003	Toronto, ON	1	0.25	0.03	1	0.12	None observed	None observed
48351	2003	Toronto, ON	1	0.25	0.008	1	0.12	None observed	None observed
48353	2003	Toronto, ON	1	0.25	0.015	1	0.12	None observed	None observed
48355	2003	Toronto, ON	1	0.25	0.015	1	0.12	None observed	None observed
48356	2003	Toronto, ON	1	0.25	0.03	1	0.25	None observed	None observed

48362	2003	London, ON	0.5	0.25	0.03	1	0.12	None observed	None observed
48426	2003	Vancouver, BC	2	0.25	0.03	1	0.25	None observed	None observed
48427	2003	Vancouver, BC	1	0.25	0.015	1	0.12	None observed	None observed
48430	2003	Vancouver, BC	1	0.5	0.03	1	0.25	None observed	None observed
48631	2003	London, ON	1	0.25	0.015	1	0.12	None observed	None observed
48873	2003	Montreal, QC	1	0.5	0.03	1	0.25	None observed	None observed
48944	2003	Regina, SK	1	0.25	0.03	1	0.12	None observed	None observed
48949	2003	Regina, SK	1	0.25	0.008	1	0.12	None observed	None observed

Cipro, Ciprofloxacin; Gati, Gatifloxacin; Gemi, Gemifloxacin; Levo, Levofloxacin; Moxi, Moxifloxacin; ND, No Data