A RAPID METHOD FOR DETECTING SINGLE NUCLEOTIDE POLYMORPHISMS USING ANTIMICROBIAL RESISTANCE IN *NEISSERIA GONORRHOEAE* AS A MODEL

BY

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABC – ATP Binding Cassette

Ala – Alanine

Arg – Arginine

Asn – Asparagine

Asp – Aspartic Acid

ATCC – American Type Culture Collection

bp(s) – Base Pair(s)

CCCP – Carbonyl cyanide m-chlorophenyl hydrazone

CDC – Centre for Disease Control and Prevention, Atlanta

CO_2 – Carbon Dioxide

Cys – Cysteine

DGI – Disseminated Gonococcal Infection

DNA – Deoxyribonucleic Acid

dNTP(s) – deoxy nucleotide triphosphate(s)

ddNTP(s) – dideoxynucleotide triphosphate(s)

EDTA – Ethylenediaminetetraacetic Acid

far – Fatty Acid Resistance

GCMB – Gonococcal Medium Base

Glu – Glutamic Acid

Gly – Glycine

HIV – Human Immunodeficiency Virus

Hr(s) – Hour(s)

IgA, IgG, IgM – Immunoglobulin A, G, M

Ile – Isoleucine

LOS – Lipooligosaccharide

LPS – Lipopolysaccharide

Lys – Lysine

MAMA – Mismatch Amplification Mutation Assay

MATE – Multidrug and Toxic Compound Extrusion

MFS – Major Facilitator Superfamily

$MgCl_2$ – Magnesium Chloride

MIC(s) – Minimum Inhibitory Concentration(s)

mtr – Multiple Transferrable Resistance

NCCLS – National Committee for Clinical Laboratory Standards

NonRI-SSCP – Non Radioisotopic Single Strand Conformation Polymorphism

PCR – Polymerase Chain Reaction

PFGE – Pulsed Field Gel Electrophoresis

PID – Pelvic Inflammatory Disease

PPNG – Penicillinase Producing *Neisseria gonorrhoeae*

Pro – Proline

QRDR – Quinolone Resistance Determining Region

RBS – Ribosomal Binding Site

RND – Resistance Nodulation Cell Division

SAP – Shrimp Alkaline Phosphatase

Ser – Serine

SMR – Small Multidrug Resistance

SNP(s) – Single Nucleotide Polymorphism(s)

ssDNA – Single stranded Deoxyribonucleic Acid

STI – Sexually Transmitted Infection

TAE – Tris Acetate EDTA

Thr – Threonine

Tm – Melting Temperature

TRNG – Tetracycline resistant *N. gonorrhoeae*

TSA – Trypticase Soy Agar

Tyr – Tyrosine

WHO – World Health Organization

ABSTRACT

Chromosomal mediated antimicrobial resistance in *Neisseria gonorrhoeae* can develop as a result of three main processes including the alteration of target enzymes, changes in transmembrane transport channels and active efflux pump function. Single nucleotide polymorphisms (SNPs) of target genes such as DNA gyrase (*gyrA*) and topoisomerase (*parC*), together with mutations in the promoter regions of the efflux pumps *norM* and *mtr* can confer resistance to the macrolides, penicillins and fluoroquinolones. These SNPs were analyzed using the SNaPshot method to allow for rapid detection of resistant isolates. Oligonucleotides were developed in the 5' to the 3' direction, ending one nucleotide adjacent to the specific SNP of interest. Single base extension reactions were performed and were detected using capillary electrophoresis. The SNaPshot procedure from Applied Biosystems employed in this study adds a single fluorescently-labelled nucleotide complementary to this SNP at the 3' end by a primer extension polymerase reaction. Then using capillary electrophoresis, the labelled nucleotide is detected, enabling differentiation between A, C, T, or G. SNP results obtained were verified using DNA sequencing and both single and multiplexed reactions were carried out to increase the efficiency of the procedure. Spiked urine samples were also observed to determine if SNPs could be detected clinically. Single reactions enabled the characterization of all confirmed and relevant SNPs. With multiplex primer extension, multiple peaks were observed, each corresponding to one of the SNPs in the gene. This technique was explored for its applicability to detect SNPs of *gyrA* and *parC* mutations. Observable SNP detection limits were seen in spiked urine

samples at 10^8 cells/mL in as early as 4 hours. DNA sequencing results confirmed the SNPs identity in each case. Thus, capillary electrophoresis using the SNaPshot protocol is another way to rapidly identify clinically resistant strains of *Neisseria gonorrhoeae.* This technique has also been shown to reduce analysis time compared to DNA sequencing and produces the same results.

1. INTRODUCTION

1.1 Description of *Neisseria gonorrhoeae*

1.1.1 Identification and Structure

The genus *Neisseria* is composed of many species. Most are non-pathogenic species that are part of the normal flora with two that are pathogenic: *Neisseria meningitidis* and *Neisseria gonorrhoeae* (11). *Neisseria meningitidis* infection involves the dissemination of the bacterium in blood or meningococcemia which can progress to the more severe meningococcal meningitis leading to increased intracranial pressure, chills, fever, malaise and vomiting (11). *Neisseria gonorrhoeae* is an obligate human pathogen that causes gonorrhea (11, 106). *N. gonorrhoeae* has become highly adapted to mucosal areas in the body, in particular to the human genital tract as its reservoir. Presumptive identification of *N. gonorrhoeae* is based on its appearance in Gram stain smears, colony morphology and a positive oxidase test (18). Fluorescent-antibody staining, coagglutination with particular antibodies, specific biochemical tests, and DNA probes can be utilized for confirmation (11).

The *N. gonorrhoeae* cocci are 0.6 to 1μm in diameter, usually seen in pairs (diplococcus) with their adjacent sides flattened (11). Structurally, the organisms possess pili, hair-like appendages extending from the cell surface, that are involved in adherence to host membranes (11). The outer membrane is composed of proteins, phospholipids and lipopolysaccharide (LPS) that is distinguished from LPS of Gram negative enteric bacteria in its highly branched oligosaccharide structure

and lack of O-antigen subunits (11, 18). For this reason, it is often referred to as lipoligosaccharide (LOS) and is a highly toxic substance (11, 18).

1.1.2 Clinical Manifestation

Neisseria gonorrhoeae can cause both symptomatic and asymptomatic genital and extragenital tract infections. Many of the symptoms are similar to those of other sexually transmitted infection's (STI's) such as *Chlamydia trachomatis*, thus it is often difficult to diagnose gonorrhea based on syndromes alone (105). For uncomplicated gonococcal infections in men, symptoms will develop 2 to 5 days post-infection with the most common complaint as urethral discharge and painful urination (105, 115). For women, the incubation period is longer at 10 days with the main symptoms of increased or abnormal vaginal discharge (105, 115). However, other symptoms may include a burning sensation or itching as well as bleeding during or after intercourse and a smelly discharge (115). In both cases, asymptomatic people are important reservoirs for transmission as well as at increased risk for developing complications (11). Complications occur more often in women with initial symptoms consisting of persistent bleeding and lower abdominal pain (115). Infections that are not successfully treated at this stage may ascend to the endometrium and fallopian tubes. At these sites, the syndrome due to infection is known as pelvic inflammatory disease (PID) resulting in endometritis, salpingitis, and periotonitis which may ultimately lead to infertility and ectopic pregnancy (11, 21, 41, 115). Disseminated gonococcal infections (DGI) result from gonococcal

bacteremia in 1 to 2% of mucosal infections, disproportionately affecting women causing rash, arthritis, and fever (105). In addition, complications enhancing the transmission of HIV has also been noted (105). The inflammatory exudate which accompanies gonococcal disease, recruits target HIV cells to mucosal surfaces (106). The viral load of HIV in semen and vaginal fluids are much higher in patients co-infected with *N. gonorrhoeae* than those without gonorrhea allowing higher risks of transmission (106).

Extragenital infections most commonly occur in the rectum, pharynx, and eyes. Rectal infections result in about one-third of women with cervical infection mostly due to autoinoculation with cervical discharge but are rarely symptomatic (11). Rectal infections also occur in homosexual men resulting from anal intercourse and are often more symptomatic (11). There are minimal differences in the symptoms between men and women ranging from purulent discharge marked with burning rectal pain, itching, tenesmus, and either blood or mucus on the stool (11, 18, 105). Pharyngeal gonococcal infection occurs predominantly in women and homosexual men practicing fellatio (18). It is usually asymptomatic with most patients having a minimal sore throat (18). Ophthalmic infections are primarily diagnosed in neonates exposed to infectious exudates in the birth canal (11). Ocular complications can be very serious leading to corneal scarring or perforation and blindness making treatment and diagnosis important (11, 41).

1.1.3 Pathogenesis

As a sexually transmitted infection, *N. gonorrhoeae* is transmitted by sexual intercourse, whereas women are more susceptible to infection than men (115). Infection is generally limited to superficial mucosal surfaces that are lined with columnar epithelium that include the cervix, urethra, rectum, pharynx, and conjunctiva (11). Gonococcal attachment is mediated mainly by pili (1) but also by Opa proteins on cell surfaces (28) and non-specific factors such as pH, surface charges, and hydrophobicity (11, 18). Attachment will only occur to microvilli of nonciliated columnar epithelial cells. The attachment to squamous cells or ciliated cells is not observed (11). After attachment, the gonococci are drawn to the surfaces of the cell by the microvilli where there undergo a process called parasite-mediated endocytosis (Figure 1). During this process the mucosal cell membrane pinches off leaving a vacuole filled with the gonococci, which then migrates to the basement membrane of the cell to undergo exocytosis (11). It is within this subepithial tissue that the gonococcus releases its pathogenic effects. The gonococci express several antigenic variants of its LOS that is sloughed off to produce mucosal damage by causing the release of proteases and phospholipases (11). The LOS also promotes the production of tumour necrosis factor (TNF), which indirectly mediates tissue damage. The antigenic variability of the gonococcus is perhaps its most important means of survival given such a restriction in host range (106). Much of this variability is due to its ability to acquire new genetic material from related organisms. Some of this genetic material encodes resistance to

different classes of antibiotics to further enable the survival of gonococcus in its host. In addition, gonococci possess autolytic enzymes which releases fragments of peptidoglycan during growth that further contribute to the intensity of the immune response (18). To evade the humoral immune response, gonococci produces an IgA protease that cleaves secretory IgA, the primary immunoglobulin active at mucosal sites (11, 18). One way the host is able to defend itself is through the activation of macrophages and polymorphonuclear cells (PMN's). In the subepithelial tissue, gonococci are susceptible to IgG or IgM antibodies marking them for opsonization by PMN's that ultimately activates the complement system to attack and destroy the gonococcal cell membrane (18). Therefore, individuals with complement deficiencies have a markedly increased risk of acquiring systemic Neisserial infections with recurring bouts of infection (11).

Figure 1: An overview of *N. gonorrhoeae* pathogenesis at the mucosal surface adapted from Baron, S., 1996 (11). *N. gonorrhoeae* diplococcus depicted as 2 grey-filled circles with surrounding spikes representing pili-like structures. LPS – lipopolysaccharide, PMN – polymorphonuclear lymphocyte, TNF – tumour necrosis factor.

1.1.4 Antibiotic Control

Proper diagnosis of bacterial sexually transmitted infections and appropriate antibiotic treatment is crucial in limiting their spread worldwide. Antibiotic treatment depends on the susceptibility of strains in the population to the therapy. Understanding antimicrobial resistance patterns is part of this process to better ensure the emergence of resistant strains can be contained and eliminated. STI patients do not usually return to see physicians, therefore, it is crucial to treat empirically using the proper antibiotics. Among the STI pathogens, *Neisseria gonorrhoeae*, has been of public health concern due to its recent increase in prevalence as an obligate human pathogen worldwide and its rising levels of antimicrobial resistance. As it becomes resilient to inexpensive, classical antibiotics, more expensive drugs are being used to resolve infections. This is particularly alarming in developing countries, where they cannot afford the rising cost of the antibiotics on top of other economic and social priorities.

In certain regions of the world resistance to anti-gonococcal antibiotics, has developed due to several misuses of therapies such as the practice of self-treatment without fully eradicating the bacteria, the non-compliance of antibiotic prescriptions by patients, and also repeated episodes of gonorrhea allowing for lateral genetic transfer of resistance moieties between bacteria (7). For these reasons anti-gonorrheal antibiotics have been characterized depending on their effectiveness (Table 1) (7).

Table 1: Anti-gonococcal antibiotic categories (7)

- Category I drugs: Universally no longer useful
  - Penicillin/Ampicillin
  - Tetracycline
- Category II drugs: Useful with varying degrees of efficacy
  - Spectinomycin
  - Kanamycin
  - β-lactam-β-lactamase inhibitor combinations
  - Trimethoprim-sulfamethoxazole
  - Thiamphenicol
- Category III drugs: Highly effective in most parts of the world
  - Third generation cephalosporins
    - Ceftriaxone
    - Cefixime
  - Fluoroquinolones
    - Ciprofloxacin
    - Norfloxacin

1.1.5 Epidemiology of Antibiotic Resistance

After Alexander Fleming's discovery of penicillin in 1929 and the advent of World War II in the 1940's, penicillin was widely prescribed for fighting sexually transmitted gonococcal infections (27, 108). Despite the dramatic decrease in gonorrhea prevalence, a strain with high-level plasmid-mediated resistance was first isolated and described in 1976 (108). It was known as penicillinase-producing *N. gonorrhoeae* (PPNG) which prompted the STI community to aggressively pursue alternative treatments. To avoid the problem of compliance, single dose treatment was advocated. Thus, the quinolones were developed and became available in the mid-1980's as a wide spectrum antibiotic for many pathogens (104, 108). From 1989 – 1993 the Centre for Disease Control and Prevention (CDC) recommended the use of two fluoroquinolones; ciprofloxacin (500 mg) and ofloxacin (400 mg) for

the primary treatment of uncomplicated gonorrhea (62, 108). Alternative regimens included enoxacin (400 mg), lomefloxacin (400 mg) and norfloxacin (800 mg) which along with the aforementioned antibiotics are still in the 1998 and 2002 CDC treatment guidelines (12, 62, 78, 108). Health Canada recommendations for adolescents and adults (except pregnant and nursing women) include ceftriaxone (125 mg intramuscularly) in a single dose, cefixime (400 mg orally) in a single dose, ciprofloxacin (500 mg orally) in a single dose and ofloxacin (400 mg orally) in a single dose (75). However, treatment with ciprofloxacin and ofloxacin should not be used if there is a possibility infection that the infection was acquired in Southeast Asia (75). Initially these drugs successfully eradicated *N. gonorrhoeae* but reports of clinical failure emerged as early as 1992 (80). The National Committee for Clinical Laboratory Standards (NCCLS) and the CDC use the standard criteria to measure clinical resistance of *N. gonorrhoeae*, minimum inhibitory concentration (MIC), in the two more commonly prescribed drugs (62, 108):

Ciprofloxacin	Intermediate resistance	MIC 0.125-0.5 μg/mL
	Resistance	MIC ≥ 1 μg/mL
Ofloxacin	Intermediate resistance	MIC 0.5-1.0 μg/mL
	Resistance	MIC ≥ 2.0 μg/mL

By using phenotyping and genotyping surveillance techniques, it has been possible to follow the spread of certain antibiotic resistant gonococcal subtypes. Phenotypic characteristics other than minimal inhibitory concentration's that have been used include auxotyping (61) and serotyping (18). Auxotrophy is the requirement for a specific nutrient which wild type strains do not need for growth

(61). This is determined by observing the inability to grow on certain culture media that lack various amino acids, vitamins and nucleic acid bases (18, 20, 105). All gonococci require cysteine for growth but may be variable in their other nutrient requirement needs (20). The most familiar association of a gonococcal auxotype with an outcome of disease is that of AHU^- (arginine, hypoxanthine, uracil requiring) in disseminated gonococcal infection's (DGI's) (60, 61, 105). Serological testing for gonococci is based on the detection of monoclonal antibodies that have been raised against the epitopes from protein I antigens of the outer membrane complex or against LOS antigens using enzyme-linked immunosorbent assays (18, 105). Thus the auxotyping/serotyping system, combining two independent tests, increases the discrimination for a phenotypic differentiation of strains. Genotyping techniques generally involve the use of plasmid profiling and pulsed-field gel electrophoresis (PFGE). Plasmid analysis allows the ability to monitor the prevalence of strains harbouring the PPNG and TRNG phenotypes and can follow the spread of these strains from different geographic areas (24). PFGE allows the resolution of large DNA fragments for the whole genome of the species to identify individual strains particularly based on the choice of the restriction enzyme digestion since different enzymes produce different grouping of isolates (99, 105). By using these techniques it has been possible to globally monitor resistant strains.

Recent (2001) global estimates by WHO suggest that there are approximately 62 million new cases of gonorrhea a year even though this number is probably much higher due to under diagnosis and under reporting (106). Disease distribution is not even. Rates can often be ten times higher or more in regions such as sub-Saharan

Africa, south and southeast Asia, the Carribean, and Latin America than it is in Western industrialized countries (105, 106). Characteristically, people with the most marginalized social and economic conditions have the highest prevalence of disease in these countries (106). In industrialized countries, rates of gonorrhea are highest in females between 15 and 19 years old and males between 20 and 24 years old (21).

Worldwide rates for antimicrobial resistance to penicillin, tetracycline, and ciprofloxacin are shown in Table 2 for representative countries. Rates for alternative therapies such as the macrolides, cephalosporins, and trimethoprim-sulfamethoxazole are not shown because of limited published material on these antibiotics. Although tetracycline is rarely used to treat gonococcal infections it is used in various locations around the world, especially in underdeveloped countries due to its availability through non-prescription routes and low cost (110). Since tetracycline is typically not recommended for treatment, high level tetracycline resistance is used more as an epidemiological marker (6). Examining Table 2 it is evident that penicillin and tetracycline resistant *N. gonorrhoeae* are prevalent worldwide with decreasing trends of susceptibility to these agents. On the other hand, high level ciprofloxacin resistance exists as a major problem in Asia with increasing problems in Europe, Australia, and the Middle East. No ciprofloxacin resistant organisms have been isolated in Africa or South America, most likely from the reduced use of quinolone antibiotics (36, 116). In North America the rates of ciprofloxacin resistance in Canada have steadily increased from 0.01% to 2.1% in a decade while in the United States clusters of resistance have arisen in Hawaii (17%)

and California (9%) (95, 121). For all geographical locations however, MIC values for ciprofloxacin are on the rise, with decreasing susceptibilities among strains. Much of this has been attributed to widespread travel and the tourism industry whereas fluoroquinolone resistant gonococci are imported from abroad especially southeast Asia where these strains are prevalent and have been rapidly increasing since the early 1990's (115). It is a general principle that once resistance reaches ≥ 5% to an individual antibiotic in a gonococcal population, it is recommended to remove it from treatment regimens in order to substitute it for another agent that is more effective (40, 106). Thus, many of the countries from Table 2 have already switched from using ciprofloxacin and related fluoroquinolones as first-line drugs to third generation cephalosporins like ceftriaxone or to spectinomycin for patients allergic to cephalosporins (31). Nevertheless these drugs are much more costly, given intramuscularly and are less readily available bringing a variety of problems to poorer nations (56). There has also been evidence of China using ceftriaxone and spectinomycin as first-line therapies, but MICs against these antibiotics are increasing which is a frightening thought considering there are no drug alternatives after resistance to these drugs develop (68).

Table 2: Prevalences and trends of penicillin, tetracycline, and ciprofloxacin resistant *N. gonorrhoeae* based on the percentage of isolates tested at sexually transmitted infection clinics in selected geographical areas.

Geographical Area	Total PenR (%)	Trend in Pen Susceptibility	Total TetR (%)	Trend in Tet Susceptibility	Total CipR (%)	Trend in Cip Susceptibility	Reference
AFRICA							
Liberia	83	-	63	-	0	-	46
Rwanda	70.5	↓	91.4	↓	0	-	116
ASIA							
China-Guangzhou	81.8	↓	-	-	72.7	↓	131
China-Zhanjiang	23.9	↓	49.5	↓	59.3	↓	68
Hong Kong	54.9	↓	-	-	97.3	↓	122, 123
India	35.3	↓	7.4	↑	67.3	↓	8, 23
Indonesia	65.5	-	99.2	-	0	-	56
Japan	29.0	↓	1.3	↑	78.0	↓	102, 103, 104, 122, 123
Philippines	86.1	↑	17	↓	54.6	↓	4, 122, 123
Thailand	-	-	-	-	25.4	↓	111
AUSTRALIA	18.0	Stable	11.4	↓	10	↓	6, 122, 123
EUROPE							
Denmark	-	-	-	-	13.2	↓	100
England	8.7	↓	-	↓	9.8	↓	40
Greenland	5	↓	0	↓	0	↓	37
Spain	16.5	Stable	32.0	Stable	9.9	↓	3, 5
MIDDLE EAST							
Israel	16	-	8	-	61	-	30, 31
Saudi Arabia	68	-	30	-	1	-	9
NORTH AMERICA							
Canada	0.02	Stable	3.2	↓	2.1	↓	87, 95
USA- Hawaii	2.7		7.4		17		85, 121
USA- Ohio	4.6	↓	12.4	↓	0	↓	44, 58, 110
CENTRAL AMERICA							
Cuba	60.8	↓	54.2	↓	0	-	73
SOUTH AMERICA							
Argentina	10	↑	58.6	↓	0	↓	25, 39
Brazil	1.2	-	76.5	-	0	-	36

↑ - increase in susceptibility, ↓ - decrease in susceptibility
Pen - penicillin, Tet - tetracycline, Cip - ciprofloxacin, R - resistance

1.2 Mechanisms of Antibiotic Resistance

Antimicrobial resistance generally develops as a result of four main processes including the production of antibiotic-degrading enzymes, the alteration of drug targets, changes in transmembrane transport channels and active efflux pump function (Figure 2). In particular, quinolone resistance in *N. gonorrhoeae* can develop as a result of three of these processes including 1) the alteration of the target enzymes (Figure 2B) 2) alterations of transmembrane drug transport (Figure 2C) and 3) active efflux pumps (Figure 2D). No specific quinolone-modifying or degrading enzymes have been found as a mechanism of fluoroquinolone resistance (53).

Figure 2: The four main mechanisms of antibiotic resistance from Putman *et al* 2000. Imagine the grey circle that encompasses all four quadrants to represent a bacterial cell membrane with the external milieu on the outside and the cytoplasm on the inside. Hypothetical antibiotics are depicted as pacman-like moieties. In quadrant A), the scissors represent drug-degrading enzymes that are capable of deactivating the antibiotics making them no longer effective. These enzymes can be produced both intra- and extracellularly. Alteration of target enzymes necessary for DNA replication, transcription, and translation is shown in quadrant B). Normal target enzymes (unmodified black bars) contain the complementary binding sites to the specific antibiotic. However, when these enzymes undergo a change in structure (black dumbells), such as through mutations, the drug can no longer recognize it as a target and does not bind. The cell is also capable of preventing the influx of drug by altering the permeability of its outer membrane shown in C). This most commonly occurs through structural changes to porins or other outer membrane proteins. If the drug is still able to pass through into the cytoplasm as shown in D), the cell has evolved efflux pumps to actively extrude the foreign agents using an energy dependent process (93).

Putman, *et al*. 2000.

1.2.1 Chromosomal mutations in target enzymes: DNA gyrase and Topoisomerase IV

Quinolones are novel antimicrobial agents because they directly inhibit DNA replication. Inhibition occurs by the interaction with complexes of DNA and two related but distinct targets within the bacterial cells, DNA gyrase, and topoisomerase IV (30, 53, 54, 62, 86, 100, 103, 104, 108). Within DNA gyrases, fluoroquinolones have shown to preferentially bind to the *gyrA* region inhibiting the enzyme from supercoiling the DNA (53). For topoisomerase IV, the *parC* subunit is targeted preventing the decatenation of the interlocking daughter DNA strands. In both cases, fluoroquinolones appear to trap the enzyme on the DNA forming a physical barrier to the movement of the replication fork resulting in cell death (53)

Fluoroquinolone resistance results from various point mutations in gyrase and topoisomerase enzyme structures rendering the quinolone ineffective at binding. DNA gyrase consists of an A subunit and a B subunit. Although mutations in *gyrB* can confer low-level resistance, high-level resistance is associated with mutations in the quinolone-resistance-determining region (QRDR) of *gyrA* between amino acids 54 to 146 (62, 104). However, the molecular nature of the interaction of quinolones with their target enzymes is only incompletely understood. In particular, a point mutation of Ser-91 to Phe is the most common alteration in highly resistant strains (MICs≥1 μg/mL) followed by Asp-95 to Gly (86, 100, 103, 104). Other point mutations identified include Ser-91 to Tyr and Asp-95 to Gly and Asn. In some Japanese studies Asp-95 mutations to Asn are more common than Gly mutations and result in MICs between 1 to 16 μg/mL (103, 104). These amino acid mutations

are clustered in α-helices near the active site tyrosine at position 122 determined by X-ray crystallography and are involved in DNA breakage (19, 54). This region is also positively charged indicating that it likely binds negatively charged DNA.

Mutations have also been observed in the *parC* region encoding topoisomerase IV. In *Escherichia coli* it is encoded by two subunits *parC* and *parE* but no *parE* homolog has been identified in *N. gonorrhoeae* (62). Topoisomerase IV is the less sensitive target to fluoroquinolone inhibition compared to the high sensitivity of DNA gyrase, making DNA gyrase the preferential target for inhibition. The difference in enzyme inhibition is believed to be due to the localization discrepancies on the bacterial chromosome. DNA gyrase is thought to be localized in front of the DNA replication fork so collisions occur more frequently after quinolone-gyrase-DNA complexes are formed (54). Whereas, topoisomerase IV is proposed to be localized behind DNA replication forks so that collisions between the complex and fork won't occur until after a subsequent cycle of DNA replication (54).

Neisseria gonorrhoeae parC is encoded within the QRDR from amino acids 66 to 119 (94). Some common point mutations include Asp-86 to Asn, Ser-87 to Arg, Asn, or Ile, Ser-88 to Pro, and Glu-91, to Lys or Gly (86, 100, 103,104). Regardless of the alteration, *parC* mutations have not been found in resistant strains without the co-existence of a *gyrA* mutation (62, 86, 100, 103, 104). Yet, *parC* mutations may be essential to acquire high-grade resistance since *gyrA* mutations alone result in minimal increases in resistance (54, 103, 108). For example, strains with mutations in *gyrA* at Ser-91 had ciprofloxacin MICs of ≥ 0.5 μg/mL but strains with mutations in both *gyrA* and *parC* had MICs ≥ 2.0 μg/mL (62). Another study showed high-level

resistance in 91% of their *N. gonorrhoeae* strains (MICs 4 to 32 µg/mL) that had the double *gyrA* mutations at amino acid positions 91 and 95 and a single or double *parC* mutation (100). They also noted no significant correlation between the number of *parC* mutations and susceptibility to ciprofloxacin. Previous work has also shown strains with the same *parC* mutation patterns but with varying levels of ciprofloxacin resistance (MICs 8 to 64 µg/mL).

1.2.2 Transmembrane Drug Transport

The passage of quinolones through the outer membranes of *N. gonorrhoeae* is crucial for antimicrobial action. Most fluoroquinolones are sufficiently small and have a negative charge that allows passage through porin proteins (52, 53). Transport is dependent on the number and properties of porin channels. Under normal circumstances, fluoroquinolones are brought into bacterial cells by an active transport process (108). During this process, porins mediate the critical role of altering the proton-motive force across the bacterial cell membrane. Mutations resulting in the loss of specific porins or functional alterations in outer membrane proteins have been associated with low-level resistance due to reduced uptake of quinolones (108). However, diffusion rate measurements suggest that a reduction in porins alone are not sufficient to account for resistance (53). Very little research has been done on quinolone transport between membranes in *N. gonorrhoeae* and thus much has been deduced from other systems such as the largest outer membrane protein, OmpF, in *E. coli* (52).

1.2.3 Efflux pumps

Amphiphilic substances can easily cross biomembranes by diffusing through both the hydrophilic and hydrophobic domains of the bilayer. It is not surprising that prokaryotes have devised mechanisms very early on to protect themselves against agents that are endowed with potentially harmful biological activities. The evolution of multidrug resistance pumps predates the clinical use of antibiotics, the development of active efflux pumps is one such mechanism that strongly modulates the activity to a large number of lethal host substances present in the various niches in which they reside (57, 113). These systems are non-specific, therefore, they can also efflux analogs of toxic substances such as dyes, detergents, free fatty acids, antibiotics and other chemotherapeutic agents (89). Given the various number and structural diversity of antibiotics, bacteria have had to evolve numerous classes of drug efflux pumps and ways to regulate them. Despite the relative limited achievements in understanding the structure-function relationship of the drug transporters, the regulation of such systems has progressed rapidly for a number of the regulatory proteins (45).

1.2.3.1 Efflux Families

Antibiotic efflux systems can be classified into five different protein families: Resistance Nodulation Cell Division (RND), Major Facilitator Superfamily (MFS), Small Multidrug Resistance (SMR), Multidrug and Toxic Compound Extrusion (MATE), and ATP-Binding Cassette Superfamily (ABC) (70). In Gram-negative organisms , efflux systems belong primarily to the RND, MFS, SMR, or MATE

families and typically share a common three-component organization with an inner membrane channel and a periplasmic accessory protein (129). By having the arrangement, the efflux complexes traverse both the inner and outer membranes and thus this facilitates the direct movement of the material from the cytoplasm into the external environment.

RND-type transporters are the major, constitutively expressed multi-component efflux pumps in Gram-negative bacteria (130). However, they are also found in eukaryotes (70). They function by having a drug-proton antiport mechanism and have an extremely wide substrate specificity (130). Members of the RND superfamily have similar but unusual features. Their structure consists of 12 transmembrane segments (TMS) or α-helices with two large hydrophilic extracytoplasmic domains between TMS 1 and 2 and TMS 7 and 8 (129). RND transporters are typically chromosomally encoded with all being multidrug transporters playing major roles in intrinsic and acquired resistance to a variety of clinically relevant antibiotics (70). The two best studied models of RND pumps are AcrAB-TolC from *E. coli* and MexAB-OprM from *Pseudomonas aeruginosa* which pump out lipophilic (penicillin G, erythromycin, fusidic acid, and rifamycin) and amphiphilic compounds (fluoroquinolones, sulfamides, rifampicin) (89, 129). The substrates have very diverse structures, many carrying either negative or positive charges. Extensive studies of substrate specificity suggest that the sole requirement for the drug to be a substrate is the presence of a hydrophobic domain capable of insertion into the phospholipid bilayer (70). However, the pump must still discriminate between its substrates and natural components of the cell membrane

which is still mostly unknown. While some pumps like MexAB-OprM encode all three components of their tripartite pumps with the gene cluster, AcrAB does not encode the outer membrane component within its operon. Instead, AcrAB recruits the TolC, multifunctional outer membrane protein that is transcribed elsewhere in the genome, to complete the functional pump (42).

Transporters in the Major Facilitator Superfamily are found in both Gram-positive and Gram-negative bacteria and higher eukaryotes, involved with the symport, antiport, or uniport of various substrates such as sugars, Krebs cycle intermediates, phosphate esters, oligosaccarhides, and antibiotics (93). These pumps are driven by the proton-motive force usually functioning as single-component transporters like with NorA of *Staphylococcus aureus*, while others in some Gram-negative bacteria like ErmAB-TolC pump in *E. coli* that function with a membrane fusion protein (ErmA) and an outer membrane protein (TolC) (70). The NorA pump belongs to the 12-TMS cluster of multidrug transporters while QacA and QacB also from *S. aureus* and ErmB belong to the 14-TMS cluster of transporters (93). Within each cluster both single and multidrug transporters occur, displaying that there is no fundamental distinction between these two classes of pumps (70).

As their names states, the Small Multidrug Resistance type transporters are the smallest of the drug efflux proteins known, driven by the proton-motive force through drug/proton antiporters (70). They are composed of about 110 amino acids residues and four transmembrane domains (70). A single charged glutamate residue within the transmembrane domain is conserved throughout the family was found to be essential for drug transport of cationic substrates but not for uncharged

substrates (129). Some of the well characterized examples of this family include EmrE in *E. coli* that can confer resistance to lipophilic cations such as tetraphenylphosphonium, methylviologen, and ethidium bromide and Smr of *S. aureus* which confers resistance mainly to antiseptics and disinfectants (70).

The MATE family are similar to the MFS family in that they contain 12 transmembrane domains. However, it does not share any sequence homology with any members of the MFS and does not have any of the signature sequences (93). Phylogenetic analysis of the MATE family revealed the presence of three distinct clusters including NorM of *Vibrio parahaemolyticus*, YdhE of *E. coli* and hypothetical efflux proteins from *Haemophilus influenzae* and *Bacillus subtilis* (93). This family has been shown to mediate resistance to cationic dyes, aminoglycosides and fluoroquinolones using an electrochemical gradient, often as Na^+, as the driving force (70).

While most bacterial multidrug transporters utilize the proton motive force or sodium for the extrusion of cytotoxic compounds, the ABC pumps are driven by the free energy of ATP hydrolysis (93). All ATP-dependent drug efflux proteins known to date are members of the ABC superfamily, or traffic ATPases that can transport sugars, amino acids, ions, drugs, iron complexes, polysaccharides and proteins (70, 93). ABC transporters require four distinct domains, two hydrophobic domains consisting of six transmembrane α-helical segments and two hydrophilic nucleotide –binding domains consisting of the ATP-binding subunits (70, 93). ABC-type drug efflux systems are not widespread in bacteria although the first, LmrA was found in *Lactococcus lactis* and is homologous to the mammalian P-glycoprotein (93). In

fact, the high sequence conservation among the ABC transporters have allowed numerous homologous associations to be identified among many species of protozoa, yeast and the *macAB* transporter of *E. coli* (70, 130).

1.2.3.2 Efflux Systems in *N. gonorrhoeae*

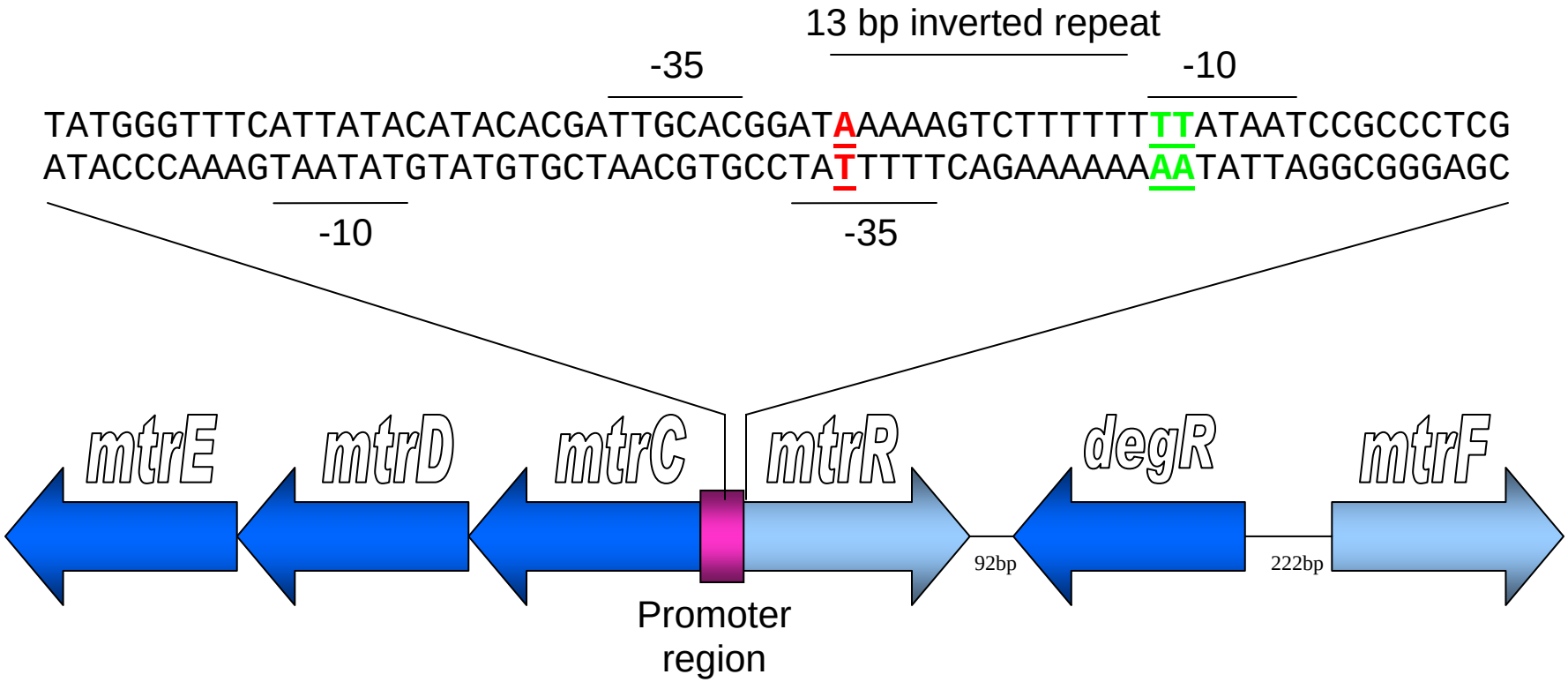
1.2.3.2.1 *mtr* (multiple transferable resistance) Regulation in *N. gonorrhoeae*

In comparison to *E.coli's* 29 putative efflux transporters, there have only been 3 identified in *N. gonorrhoeae*. Of these, the *mtrCDE* regulatory system has been the one most studied. It is similar to the MexAB-OprM pump of *P. aeruginosa* and the AcrAB-TolC system in *E. coli* (119). It is a multidrug resistant pump (MDR) that is capable of effluxing various substrates including acriflavine, crystal violet, macrolides, penicillin, fatty acids, bile salts and gonadol steroids (48). Transcription of *mtrCDE* can be increased by Triton X-100, which is a substrate similar to many hydrophobic agents (45). Therefore, many other hydrophobic agents are likely to act as natural inducers similarly to the phenomenon that occurs in quorum sensing (45). The *mtr* operon produces 3 structural proteins, MtrC, MtrD, and MtrE. *MtrC* encodes a protein belonging to the membrane fusion protein family that spans the periplasmic membrane, *mtrD* encodes an inner membrane transporter of the RND family and *mtrE* encodes an outer membrane protein that acts as a channel for export to the extracellular fluid (32, 48). From numerous studies, mutations in any of these genes render gonococci hypersensitive to several structurally diverse hydrophobic agents (32, 48, 117, 119). Divergently transcribed from *mtrCDE* is MtrR, which is a

repressor that binds to the *mtrCDE* promoter (Figure 3). It was shown that deletions in the *mtrR* gene, its binding site or a single base pair deletion within an inverted repeat in the *mtrR* promoter region resulted in increased expression of *mtrCDE* and therefore increased levels of resistance to hydrophobic agents since the regulatory actions of MtrR were dissipated (47).

In addition, the single-base pair deletion in the *mtrR* gene caused a three-fold higher increase in the *mtrCDE* expression than the deletion in the *mtrR* gene. The significant difference was hypothesized to be due to the shortening of a RNA polymerase binding domain between –10 and –35 regions of the promoter, while the truncated MtrR protein still had some effect. Recently, *mtrF* has been identified to be involved in the *mtrCDE* efflux system, not as a core component but as a possible accessory factor to help the MtrCDE pump promote resistance when exposed to high levels of hydrophobic agents (118). The exact function of MtrF has not been elucidated but it's suspected that it may have a transient structural role by stabilizing MtrCDE or by delivering the hydrophobic agents or energy requirement components to the pump.

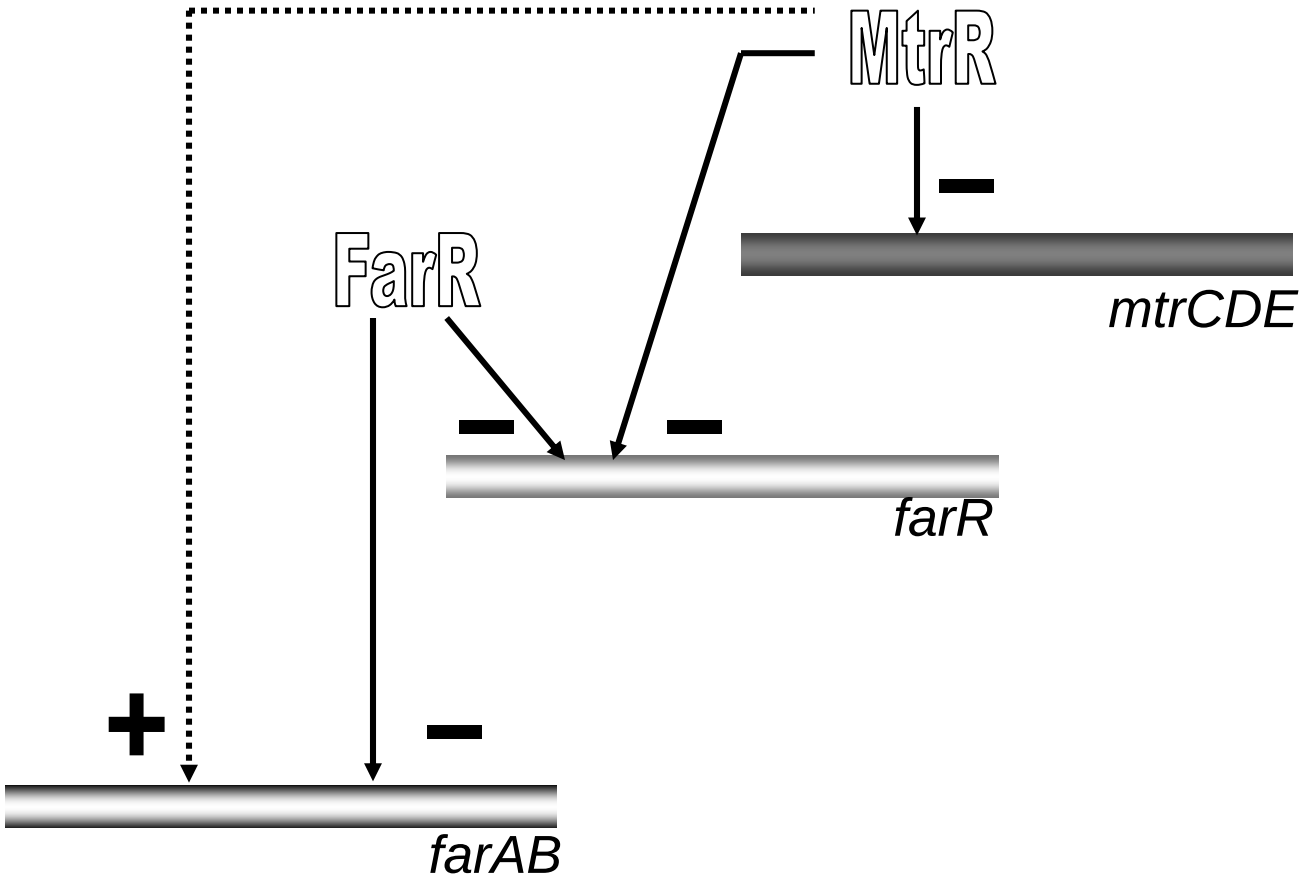
Figure 3: Schematic representation of the *mtrCDE* operon. Not drawn to scale. A 63 base pair sequence of the promoter region of *mtrR* (in pink) has been highlighted to show where mutations are present. The dinucleotide insertion is shown in green while the single base pair deletion within the 13 bp repeat sequence is shown in red. –10 and –35 base pair promoter regions are also shown for both strands. Arrows indicate the orientation of the genes and their transcription direction.


13 bp inverted repeat
-35
-10
TATGGGTTTCATTATACATACACGATTGCACGGATAAAAAGTCTTTTTTTTATAATCCGCCCTCG
ATACCCAAAGTAATATGTATGTGCTAACGTGCCTATTTTTTCAGAAAAAAAATATTAGGCGGGAGC
-10
-35
mtrE
mtrD
mtrC
mtrR
degR
mtrF
92bp
222bp
Promoter
region

1.2.3.2.2 farAB (fatty acid resistance) Regulation in *N. gonorrhoeae*

The *farAB* efflux system bears similarity to the *emrAB* system in *E. coli* and *vceAB*-encoded pump in *Vibrio cholerae* (129). The *far* system is composed of the FarA membrane fusion protein and the FarB cytoplasmic membrane transporter protein (67). The operon does not encode for its own outer membrane protein but instead utilizes *mtrCDE*'s MtrE protein to form a tripartite complex (66). Resistance from the system is to long chain fatty acids including linoleic acid, palmitic acid and oleic acid that normally have antigonococcal action as toxic fecal lipids (66). Therefore, this pump is expected to exert its effects in rectal infections where these fatty acids are in highest concentrations. Regulation in FarAB is under control of *farR*, which represses *farAB* when over expressed. Recently, it was shown that *mtrR* can indirectly regulate *farAB* by controlling the expression of the repressor encoded by *farR* (Figure 4) (67). The ability of the MtrR repressor to regulate both the *mtr* and *far* operons in opposite ways highlights an important part of bacterial adaptation and survival under stress conditions because the loss of *mtrR* results in increased expression of *mtrCDE* but decreased expression of *farAB* (67). The reverse is true if the *mtrR* repressor is left in tact. This regulatory scheme is very similar to the major porins OmpF and OmpC in *E. coli* that determine membrane permeability. In this case, OmpF, forming the larger pore of the two, is modulated by phosphorylation in response to environmental conditions as part of a two-component signal transduction system (67).

Figure 4: Regulation of the MtrR and FarR repressors. MtrR has both positive and inhibitory regulatory effects on *N. gonorrhoeae* efflux systems while FarR has both inhibitory effects on the *farAB* operon and a negative feedback function on its own production (adapted from 67).



1.2.3.2.3 *norM* Regulation in *N. gonorrhoeae*

Recently, a new efflux system has been discovered in *N. gonorrhoeae*, *norM*, that can pump out the fluoroquinolones ciprofloxacin and norfloxacin (94). The NorM pump has high homology to a Na^+-coupled multidrug efflux pump from *Vibrio parahaemolyticus* with the same name, as well as with a homolog from *E. coli*, YdhE (82). All of these Na^+-drug antiporters belong to the multidrug and toxic compound extrusion (MATE) family of transporters with twelve transmembrane domains (82, 94). The homologous region is a protein sequence GKFGXP found within the vicinity of transmembrane domains five and six (94). Although it's part of the MATE family its mechanism is similar to that of the *mtr* efflux pump. In the *norM* system, an A to G mutation in the promoter region 7 bp upstream of the *norM* ATG start site resulted in a TGAA-to-TGGA alteration of the putative ribosome binding site (RBS) in addition to another mutation in the –35 bp promoter region (CTGACG-to-TTGACG) (94). The presence of both these point mutations in conjunction with *gyrA* and *parC* mutant strains (high-level resistance) increased the resistance of gonococci to the same compounds including ethidium bromide, acriflavine hydrochloride, 2-N-methylellipticinum, and berberine as was seenin *V. parahaemolyticus.* In addition there was a two-fold increase in MIC's to norfloxacin and ciprofloxacin (94). Furthermore, cationic substrates are essential for NorM recognition.

1.3 Detection of SNPs Involved in Resistance

Molecular methods such as PCR are becoming increasingly utilized in Public Health Laboratories for infectious disease diagnosis resulting in clinical specimens not being routinely cultured for isolates (15). Therefore, novel molecular methods need to be developed for the detection and surveillance of important phenotypic traits such as antibiotic resistance (15). Various methods have been reported for the detection of single nucleotide polymorphisms in genes involved in conferring antibiotic resistance including direct sequencing, sequence-specific oligonucleotide probe hybridization using microarrays (14), mismatch amplification mutation assay (MAMA) PCR (101), and non-radioisotopic single-strand conformation polymorphism (nonRI-SSCP) (22). Each technique has been proven useful but each has its advantages and pitfalls. There are many other proven techniques but for the sake of brevity only these will be discussed here.

Direct DNA sequencing is the preferred method for discovering and screening most single nucleotide polymorphisms (SNPs), but it is not cost effective for routinely screening large numbers of samples for known SNPs (17, 69, 96).

1.3.1 Microarrays

Microarray technology is a promising tool to screen large number of microbial strains for genetic polymorphisms. Mostly this is because of its ability to perform a multitude of tests simultaneously. It currently requires 5 components: 1) the

microarray chip itself with its special surface, 2) the device for producing microarrays by spotting the nucleic acids onto the chip, 3) a fluidic system for hybridization to target DNA, 4) a scanner to read the chips, and 5) sophisticated software programs to quantify and interpret the results (14). In addition, tools are needed to extract high quality nucleic acids from biological material to prepare them for microarray use. Many of the gene collections and PCR product libraries of cDNA are available commercially for making microarrays. This technology has widely been used for large-scale genotyping of organisms looking at SNPs, such as in human cancer patients. This has been used diagnostically for identifying small insertions and deletions in the BRCA1 and BRCA2 oncogenes, which can indicate relative risk of obtaining a breast cancer phenotype in women (14). Another application for cancer research is looking at the effectiveness and toxicity of various chemotherapy drugs on a group of cells to compare gene expression profiles (50). Applying this concept to examine antibiotic effects on prokaryotes is underway. These types of experiments can help researchers identify new drug targets for increasingly resistant strains. Microarrays have moved out of specialty laboratories and into mainstream laboratories despite being fairly expensive, because they are highly effective. For instance, Ng *et al.* has applied microarray technology for identifying SNPs involved fluoroquinolone resistance in *N. gonorrhoeae* (86). Short, specific oligonucleotide probes were designed that would perfectly match the following point mutations in *gyrA*, Ser-91-Phe, Asp-95-Asn, Asp-95-Gly, and Asp-95-Ala and within *parC*, Asp-86-Asn, Ser-87-Asn, Ser-87Arg, Ser-88-Pro, Glu-91-Arg and Glu-91-Lys that were confirmed by DNA sequencing (86). Probes were also designed for wild-type

sequences of *gyrA* and *parC* to act as controls and probes that had combinations of mutations and wild type sequences together were also considered. Positive signals were noted for strains with specific probes containing the respective mutation but not others. For example, a strain that had the mutation Asp-95-Asn within *gyrA* hybridized with the *gyrA* probe Asp-95-Asn to produce a signal, but not with Asp-95-Gly or any probe. This system could easily be adapted to detect more targets and be performed at higher throughput as new mutations are discovered. One disadvantage is that only mutations that have a particular probe designed for them can be detected and areas such as QRDRs with several adjacent mutations require several probes that incorporate both wild type and mutated sequences, potentially making analysis difficult with so many possible hybridization combinations.

1.3.2 Mismatch Amplification Mutation Assay (MAMA PCR)

Another novel technique for identifying resistance mutations was adapted by Zirnstein *et al.* 1999 called mismatch amplification mutation assay (MAMA) PCR. Briefly this method involves designing a forward conserved primer and a reverse mutation detection primer that when used in a PCR reaction with a mutant strain generates a certain base pair product that is a positive indication of the mutation. When wild type or fluoroquinolone-susceptible DNA was used in this reaction, no amplification occurs and when a PCR with two conserved primers was performed with either wild type or mutated DNA to produce a positive PCR control. MAMA PCR was originally described for identifying a threonine to isoleucine change in

codon 86 of the QRDR or *gyrA* in *Campylobacter jejuni* (132) and a homologous mutation in *Campylobacter coli* (133). Recently it was adapted for use to identify mutations within codons 91 and 95 in the *gyrA* QRDR for *N. gonorrhoeae* (101). Three sets of primers were developed; two conserved *gyrA* primers for a positive control, a set for detecting the mutation at codon 91 of Ser to Phe with both a conserved primer and one containing the mutated sequence, and the final set of mismatched primers for identifying the Asp to Gly mutation at codon 95 (101). A primer set was not designed to identify the Asp to Asn mutation at codon 95. Using this technique all 25 isolates tested that had the Ser-91-Phe mutation and all 15 isolates with the Asp-95-Gly mutation as confirmed by DNA sequencing could be detected. However, one false positive result was found, wrongly classifying a strain with an Asp-95-Asn mutation as having the Asp-95-Gly mutation (101). Therefore, this is among one of the limitations of this assay. Other disadvantages is that all of the work done thus far on MAMA PCR has looked at only one or two mutations of interest. Whether it can be applied to multiplex multiple mutations together for increased throughput or identify mutations that are within close proximity of each other has yet to be examined. In addition, new primer sets need to be designed for each mutation and MAMA PCR can only be used for identifying the particular mutation that the primer is made for. On the other hand, it is a much simpler, more specific, rapid, inexpensive and portable method than DNA sequencing that can easily be incorporated into any laboratory that has a Thermal Cycler.

1.3.3 Non Radioisotopic Single Strand Conformation Polymorphism

Another method that has been utilized for SNP detection is non-radioisotopic single stranded conformation polymorphism or non-RI-SSCP. This procedure was designed to identify several point mutations within the QRDR of *gyrA* in *C. jejuni* (22). These mutations in particular were at codons 70 (Ala-Thr), 86 (Thr-Ile), and 90 (Asp-Asn). The process begins with an initial PCR of the *gyrA* QRDR followed by the denaturation of these products using a solution containing 95% deionized formamide, 20mM EDTA, 0.05% xylene cyanol, and 0.1% bromophenol blue (22). The resulting single stranded DNA is run on a polyacrylamide gel and silver stained. The concept states that the various mobility shifts and separation of bands depends on the altered folding structures of the ssDNA due to mutations (22). So for each mutation a different mobility pattern is seen. This technique is simple, rapid and gives a specific electrophoretic profile for each mutation allowing it to be used as an epidemiological marker. However, it is sometimes difficult to distinguish two profiles apart given the minor differences in band positions, so positive controls containing known mutations must be used for comparison.

1.3.4 SNaPshot

A novel technique, utilizing a single base extension reaction called SNaPshot is detectable by capillary electrophoresis, has been used extensively in characterizing human genome SNPs implicated with diseases such as cancer,

Alzheimer's disease, and diabetes as well as SNPs in spongiform encephalopathies (13, 26, 34, 64, 69, 76, 88, 90, 96, 112, 134). However, this technology has been slow to advance into prokaryotic studies, with the majority of reports focusing on high-throughput fingerprinting for the construction of large bacterial genome maps (74). In the SNaPshot method for detecting SNPs, an oligonucleotide primer is designed so that its 3' end is positioned directly next to the SNP of interest (16). Fluorescently labelled ddNTP's are then used in a base-pair extension reaction that insert into the SNP site complementary to the template strand and therefore act as chain terminators to identify the mutation, if present. Up to 96 samples are then loaded onto a capillary electrophoresis instrument such as the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) that separates the oligonucleotides in a polymer sieve based on size and the fluorescently labelled bases are visualized through a detection window near the end of the capillary (16, 81, 112). A unique application of SNaPshot is that it can be multiplexed in order to detect many mutations in a single reaction allowing high throughput genotyping at a very low cost.

1.4 Thesis Objectives: A Rapid Detection Method for Analyzing SNPs Involved in Antimicrobial Resistance using *Neisseria gonorrhoeae* as a Model

In previous work done by Ng *et al.*, two strains with identical *gyrA* and *parC* mutation profiles were identified but varied in their MIC values (Table 3) (86).

Table 3: Characterization of two strains based on their identical *gyrA* and *parC* mutations but variable MIC values to ciprofloxacin.

Strain	Cipro MIC	*gyrA*		*parC*		
	(µg/mL)	Ser-91	Asp-95	Asp-86	Ser-87	Ser-88
19703	16	Phe	Gly	-	Arg	-
21146	4	Phe	Gly	-	Arg	-

The 4-fold increase in resistance in MIC between strains 21146 and 19703 lead us to believe besides the mutations of *gyrA* and *parC,* other mechanisms may be causing the increase in resistance to ciprofloxacin. Since the efflux systems in *N. gonorrhoeae, mtrCDE* and *farAB* have not been shown to pump out fluoroquinolones we hypothesize that there is a novel efflux pump involved in pumping out fluoroquinolones thus causing decreased susceptibility. In 1998, Morita *et al.* discovered the *norM* pump in *Vibrio parahaemolyticus* that was shown to pump out the cationic substrates ciprofloxacin and norfloxacin (82). In February 2003, Rouquette-Loughlin *et al.* confirmed that NorM was a novel efflux pump in *N. gonorrhoeae* and that it needed cationic substrates to function (94). They also showed that this pump could extrude ciprofloxacin. The two key mutations that they elucidated in the *norM* promoter region raised MICs by 2-fold similarly to what we had noticed with our strains. Therefore, our first objective was to see if *N.*

gonorrhoeae strain 19703 possessed these mutations which may have been the reason for the increase in resistance. We wanted to determine this by performing sequencing analysis of the *norM* gene and if mutations were present to confirm that they were responsible for the resistance by transformational studies. We found that *N. gonorrhoeae* strain 19703 did not possess the known mutations in *norM*, but did contain a novel deletion in the *norM* promoter region. Transformational studies were used to confirm if the novel mutation was involved in the increased resistance as were efflux accumulation studies using the proton motive force uncoupler carbonyl cyanide m-chlorophenyl hydrazone.

Our second main objective was to design a rapid detection method to identify clinically relevant instances of *N. gonorrhoeae* resistance due to mutations in *parC, gyrA, mtrR*, and *norM*. Presently the gold standard for detecting these mutations is by sequence analysis which can be labour intensive and time consuming. The SNaPshot technique trade marked by Applied Biosystems can rapidly identify single nucleotide polymorphisms with the use of a Genetic Analyzer. Oligonucleotides ranging from 16 to 50 nucleotides in length were designed for published mutations in the *parC, gyrA, mtrR*, and *norM* genes to better understand and predict the level of antimicrobial resistance in each strain tested. The oligonucleotides were also optimized for multiplex use for establishing a superior comprehensive detection of the various mutations while reducing the costs for expensive reagents. This technology was applied to detecting specific SNPs in *N. gonorrhoeae* as a model organism but can also be developed and applied for use of other SNPs of interest from different organisms. Additionally we sought to examine the applicability and

sensitivity of this technique to detect *N. gonorrhoeae* SNPs in clinical samples. Thus, the urine from three volunteers was collected and spiked at various concentrations to determine detection parameters.

2. MATERIALS AND METHODS

2.1 Bacterial Strains and Culture Conditions

All of the strains in this study were obtained from the National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg as part of a national surveillance program. The *N. gonorrhoeae* strains used were ciprofloxacin susceptible (MIC < 0.255µg/mL), intermediate resistant (MIC 0.25-0.5µg/mL) and resistant (MIC ≥ 1 5µg/mL), with minimum inhibitory concentrations (MICs) ranging from 0.004 to 32 µg/mL. *N. gonorrhoeae* strain FA1090 was used as a wild type strain with a ciprofloxacin MIC of 0.004 µg/mL. Antimicrobial susceptibility testing and auxotype profiling was performed using an agar dilution method as previously described (86). The *N. gonorrhoeae* and *Moraxella osloensis* ATCC 11976 strains were subcultured onto GC medium base (GCMB) (Difco Laboratories, Detroit, MI) containing 0.2% BioX (Quelab, Montreal, PQ) and incubated in a 5% CO_2 atmosphere for 24 hours at 35˚C. Negative control strains of *S. aureus* ATCC 29213, *Lactococcus lactis*, *Lactobacillus acidophilus* ATCC 314 and *E. coli* ATCC 35218 were subcultured onto TSA 5% sheep blood agar and incubated in a 5% CO_2 atmosphere for 24 hours at 35˚C. All culture stocks were maintained in BHI containing 20% glycerol and stored at -80˚C until needed.

2.2 PCR Amplification

Table 4 indicates the primers pairs used for the amplification process. The PCR reactions were performed in a thermocycler, (Geneamp PCR system 9700 Applied Biosystems) with a total volume of 50 µL reaction mix containing 1 x PCR

buffer (diluted from 10x, Invitrogen), 1.5 mM $MgCl_2$, 1 µM of each primer pair, 0.2 mM dNTP's, and 0.5 U Taq DNA polymerase, recombinant (Invitrogen, Carlsbad, CA). Thermocycling conditions were 94˚C for 5 minutes followed by 35 cycles of 94˚C for 50 seconds, 56˚C for 50 seconds, and 72˚C for 2 minutes, ending with a 7 minute incubation at 72˚C.

Table 4: Primers used for PCR amplification

Primer	Primer Type	Sequence	Length of product
GyrA-F3	Forward	5' ACTGTACGCGATGCACGAGC	266bp
GyrA-R2	Reverse	5' TCTGCCAGCATTTCATGTGAG	
ParC-left	Forward	5' GTTTCAGACGGCCAAAAGCC	331bp
ParC-right	Reverse	5' GGCATAAAATCCACCGTCCCC	
Mtr-F1	Forward	5' TGCAGTCTCAATTTTATGGGTTTC	163bp
Mtr-R1	Reverse	5' AAGGCGGCAAGCATCAGG	
NorM-F1	Forward	5' AAACGGCGATGCTTCCTTC	582bp
NorM-R1	Reverse	5' AAATCATGCCGAAAATCCCC	
NorMup-F1	Forward	5' GATGCTCTGTAAACGGCGATGCTTCCTTC	592bp
gyrA-F2	Forward	5' CCTTTGTTTTTGCGGCTGTAATC	480bp
gyrA-R2	Reverse	5' CCGCGCAGGGAATGGTG	
gyrA-F3	Forward	5' CGCAGCGCCGACGAGGTAG	409bp
gyrA-R3	Reverse	5' AAACCCAGCCGACCACCGAC	
gyrA-F4	Forward	5' AGGTTGGTAAAACACATCAAATAGT	539bp
gyrA-R4	Reverse	5' CCTCGTTGAAGAAATGCTGAC	
gyrA-F5	Forward	5' CCTTCCGGACGCATCATT	535bp
gyrA-R5	Reverse	5' GTTATCGACGAAATCCCCTATCA	
gyrA-F6	Forward	5' GTTTAAGACGACTTCGGCATTTTC	413bp
gyrA-R6	Reverse	5' ATCCCGCCGCACAACCT	
gyrA-F7	Forward	5' AGTCGGGGGCTTGGATAATGT	464bp
gyrA-R7	Reverse	5' CGATGCACGAGCTGAAAAATAAC	

2.3 DNA isolation and DNA sequencing

Sequencing was used to initially examine the *norM* gene for mutations and to sequence subsequent transformants. Regions containing the identified SNPs were also sequenced in various strains to determine the validity of the SNaPshot technique. Crude DNA was isolated from strains by boiling a small loopful of cells in

200 µL of autoclaved, deionized water for 10 minutes. Two µL of this DNA was used as template to PCR amplify the regions produced from the primers in Table 4. The PCR products were separated on a 1xTAE, 1.5% low melting point agarose gel (Invitrogen, Carlsbad, CA) and stained with 5% ethidium bromide (FisherBiotech, Fair Lawn, NJ). The band of interest was extracted from the agarose gel compared to a 100 bp molecular size marker (Invitrogen, Carlsbad, CA) and was purified using a DNA purification kit (Promega PCR prep kit, Fisher, Nepean, ON). The concentrations of purified DNA were determined using a Nanodrop ND-1000 Spectrophotometer (Coleman Technologies, Wilmingon, DE). The purified amplicons were sequenced using the specific primers (Table 4) by the DNA Core Facility at the National Microbiology Laboratory. Sequencing results were aligned by Clustal W based on their loci to confirm point mutations in various strains.

2.4 Transformation Studies

Genomic DNA from clinical *N. gonorrhoeae* strains 19081, 19703, and 21146 was introduced into piliated *N. gonorrhoeae* reference strain FA1090 (ciprofloxacin MIC of 0.004 µg/mL) by transformation. Briefly, an overnight culture of piliated FA1090 was suspended in 2 mL of diluent buffer (10% salts solution supplemented with 0.54% KH_2PO_4 and 0.05% Bovine Serum Albumin, pH 6.9) to a McFarland standard of 0.5, inoculated on GCMB, and incubated for 1 hr at 37˚C in an atmosphere enriched with 7% CO_2. Piliated cells (T1 and T2) were distinguished by the following criteria: produced small colonies, usually tan in colour, that were raised on the plate and were easy to move with a loop. When oblique light was shone on

the agar using a dissecting microscope (100x) (Leica Mikroskopie & Systeme Gmbtt Wetzler, Germany), a small pit could be seen. Fresh type 2 colonies had dark rings around their perimeters while type ones were similar but without the ring compared to types 3 and 4 (nonpiliated) which tended to spread out flat on the agar, were more white in colour and did not have dark rings (35).

Table 5: Oligonucleotides used to detect SNPs

Gene Target	Name[a]	Sequence	Length (bp)
gyrA	91AF	5' ATACCACCCCCACGGCGATT	20
	95AF1	5' GTAAATACCACCCCCACGGCGATTCCGCAGTTTAC	35
	95AF2	5' GTAAATACCACCCCCACGGCGATTTCGCAGTTTAC	35
	95AR	5' ACTGACTGACTGACTGGAAATTTTGCGCCATACGGACGATGGTG	48
parC	86CF	5' TGGGTAAATACCATCCGCACGGC	23
	87CF1	5' CGTGGTCGGCGAGATTTTGGGTAAATACCATCCGCACGGCGAC	43
	87CF2	5' CGTGGTCGGCGAGATTTTGGGTAAATACCATCCGCACGGCAAC	43
	87CR1	5' ATCCTGAGCCATGCGCACCATCGCCTCATAGGCGGAA	37
	87CR2	5' ATCCTGAGCCATGCGCACCATCGCCTCATAGGCGGGA	37
	88CR	5' CCTGAGCCATGCGCACCATCGCCTCATAGGCGG	33
	91CF1	5' CATCCGCACGGCGACAGTTCCGCCTAT	27
	91CR2	5' CCTGAGCCATGCGCACCATCGCC	23
mtrR	MA	5' CATTATACATACACGATTGCACGGATAAAA	30
	MT	5' CGGTTTGACGAGGGCGGATTATAAAAAA	28
norM	norM	5' TATTTCTCATCACTTCCCGCTGCCGTTTTTTT	32
	NorM-10	5' TCATCACTTCCCGCTGCCGTTTTTTT	26
	NorM-15	5' ATTTCTCATCACTTCCCGCTGCCGTTTTTTT	31
	7N	5' CGAGCAGCATAAGTGT	16
	7N	5' CGGCATTTTTATTGACTG	18
	7N	5' TTTTGTTTCAGACGGCATTTTTATTGACTG	30
	7N	5' GGAAAAGGAAAAGCGGTCGAGGTCGAGCAGCATAAGTGT	33
	7N	5' TTCAGGAAGACGGAAAAGGAAAAGCGGTCGAGGTCGAGCAGCATA AGTGT	50
	35N	5' ATACCCCGTATCCGCCGT	18
	35N	5' TATACCCCGTATCCGCCGT	19
	35N	5' AGTAAAAAATACCGTGCCGTCA	22
	35N	5' CATTATATAGTAAAAAATACCGTGCCGTCA	30
	35N	5' CTGAATATACTTTATATACCCCGTATCCGCCGT	39

[a] The oligonucleotides were named first by the codon or region in which the SNP occurs, followed by A, C, M, or N related to the *gyrA*, *parC* , *mtrR* or *norM* genes respectively, and then by the direction they were designed on the template DNA, either F (forward) or R (reverse). The 1 and 2 signifies alternate oligonucleotides that had to be designed due to redundant mutations.

Amplified DNA (1µg) using the NorM forward uptake primer (NorMup-F1) and NorM reverse primer (NorM-R1) (Table 4) was then applied to the inoculated plate. Goodman and Scocca, 1988, identified a 10 bp sequence (GATGCTCTGT) that is required for the specific uptake of gonococcal DNA from the environment (43). Therefore, DNA with this uptake sequence will preferentially be chosen in the transformation process and is necessary to enable the gonococcus to transport DNA molecules into the cell (Stein 1991). After a 24 hr incubation period, individual colonies from the GCMB plates were transferred to GCMB containing ciprofloxacin (0.004, 0.008, 0.016, 0.032, 0.064, 0.125, 0.25, 0.5, 1.0 µg/mL) (compliments of Bayer, Etobicoke, Ontario). Potential transformants were again subcultured onto GCMB containing the respective concentration of ciprofloxacin prior to MIC determination and sequence analysis of the *norM*, *gyrA*, and *parC* genes.

Liquid transformations were also performed as an alternate method essentially as described by Hill *et al* (51). Briefly, T1 and T2 piliated gonococci from reference strain FA1090 were swabbed from an 18 hr culture and resuspended to an approximate density of 10^8 cells/mL (a McFarland standard of 0.5) in liquid GCMB medium (with the same composition of GCMB except for agar) (adapted from 65). DNA from gonococci strains 19703 and 21146 containing the *norM* uptake sequence (Table 4) were added to separate cell suspensions at a concentration of 1 µg/mL, and the suspensions were incubated for 30 min at 37˚C and 7% CO_2 prior to being diluted 1:10 with pre-warmed medium. The cells were then incubated a further 4 hr before being plated on GCMB medium with ciprofloxacin concentrations ranging from 0.004 µg/mL to 1.0 µg/mL. Plates were incubated at 37˚C and 7% CO_2

overnight or until colony growth was observed. Potential transformants were again subcultured onto GCMB containing the respective concentration of ciprofloxacin prior to MIC determination and sequence analysis of the *norM*, *gyrA*, and *parC* genes.

2.5 Inhibition of Efflux Pump

Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) (Sigma-Aldrich Co, St. Louis, MO) was used to determine the effect of dissipating proton gradients on ciprofloxacin MIC's in numerous *N. gonorrhoeae* strains. Various concentrations of CCCP (0.25, 0.5, 2.5, 5.0, 12.5, 25.0 μM) dissolved in acetone were incorporated into GCMB plates containing dilutions of ciprofloxacin of 0.004 μg/mL up to 16 μg/mL. Antimicrobial susceptibility testing was performed using an agar dilution method as previously described (86). Control plates were included using acetone without CCCP for each concentration of ciprofloxacin to ensure the acetone was not toxic to cells.

2.6 Design of SNaPshot extension primers

Table 5 shows the sequences of the oligonucleotides used for SNP detection. Figures 5, 6, 7 and 8 show the positions of oligonucleotides with respect to their positions on the wild type reference strain FA1090 DNA sequence. In designing the primers, the position of the oligonucleotides is restricted, as they must be designed to bind immediately adjacent to the SNP. Typically, Tm's of 50°C were optimal but by varying the oligonucleotide length this parameter was not always possible.

Binding to either the sense or antisense strand will lead to different base pairs incorporated and hence varying peaks will result. Therefore, while multiplexing it is useful to design the oligonucleotide primers for different strands to prevent overlapping of closely associated primers. It is also important to vary the length of the primer in order to maximize the number of simultaneous products that can be detected. Adjusting the primer binding site and the length of the primer, made it possible to simultaneously detect up to six SNPs per reaction using up to eight oligonucleotides. Alternate oligonucleotides had to be made for *gyrA* at codon 95 and *parC* at codons 87 and 91 to compensate for redundant mutations flanking these regions (Figures 5B, 6B). All oligonucleotides were made and salt purified by Integrated DNA Technologies Inc., Coralville, IA.

Figure 5: Schematic diagram showing the design of oligonucleotides for *gyrA* SNPs using wild type strain FA1090 DNA. A) 266 bp sequence of *gyrA* with SNPs highlighted in yellow. Sequence highlighted in grey is blown up in panel B as double stranded DNA. B) *gyrA* codons 86 to 101 showing the positions of 4 oligonucleotides 91AF, 95AF1, 95AF2, and 95AR with the arrow tip ending on the SNP. 91AF, 95AF1, and 95AF2 were designed on the forward strand with 95AF1 and 95AF2 differing by one nucleotide: 95AF1 containing the wild type nucleotide and 95AF2 containing the mutated SNP. 95AR was designed on the reverse strand. Blue arrows identify guanosine (G) residues, yellow arrows identify cytosine (C) residues and red arrows identify thymidine (T) residues.

A

gyrA

ACTGTACGCGATGCACGAGCTGAAAAATAACTGGAATGCCGCCTACAAAAAATCGGCGCGCATCGTCGGCGACGTCATCGGTAAATACCACCCCCACGGCGATTCCG
CAGTTTACGACACCATCGTCCGTATGGCGCAAAATTTCGCTATGCGTTATGTGCTGATAGACGGACAGGGCAACTTCGGATCGGTGGACGGGCTTGCCGCCGCAGCC
ATGCGCTATACCGAAATCCGCATGGCGAAAATCTCACATGAAATGCTGGCAGA

B

Figure 6: Schematic diagram showing the design of oligonucleotides for *parC* SNPs using wild type strain FA1090 DNA. A) 331 bp sequence of *parC* with SNPs highlighted in yellow. Sequence highlighted in grey is blown up in panel B as double stranded DNA. The 2 highlighted nucleotides in yellow not within the sequence highlighted in grey are silent mutations and thus were not considered in this study. B) *parC* codons 80 to 95 showing the positions of 8 oligonucleotides 86CF, 87CF1, 87CF2, 87CR1, 87CR2, 88CR, 91CF1, and 91CR2 with the arrow tip ending on the SNP. 86CF, 87CF1, 87CF2 and 91CF1 were designed on the forward strand with 87CF1 and 87CF2 differing by one nucleotide: 87CF1 containing the wild type nucleotide and 87CF2 containing the mutated SNP. 91CF1 had to be designed overlapping 4 SNPs. In this case, the 4 wild type nucleotides were incorporated in this oligonucleotide. 87CR1, 87CR2, 88CR and 91CR2 were designed on the reverse strand with 87CR1 and 87CR2 differing by one nucleotide: 87CR1 containing the wild type nucleotide and 87CR2 containing the mutated SNP. 87CR1, 87CR2 and 88CR had to be designed overlapping 2 SNPs. In this case, the 2 wild type nucleotides were incorporated in these oligonucleotides. Blue arrows identify guanosine (G) residues, yellow arrows identify cytosine (C) residues, green arrows identify adenosine (A) residues, and red arrows identify thymidine (T) residues.

A

parC

TTTCAGACGGCCAAAAGCCCGTGCAGCGGCGCATTTTGTTTGCCATGCGCGATATGGGTTTGACGGCGGGGGCGAAGCCGGTGAAATCGGCGCGCGTGGTCGGCGAG
ATTTTGGGTAAATACCATCCGCACGGCGACAGTTCCGCCTATGAGGCGATGGTGCGCATGGCTCAGGATTTTACCTTGCGCTACCCCTTAATCGACGGCATCGGCAA
CTTCGGTTCGCGCGACGGCGACGGGGCGGCGGCGATGCGTTACACCGAAGCGCGGCTGACGCCGATTGCGGAATTGCTGTTGTCCGAAATCAATCAGGGGACGGTGG
ATTTTAT

B

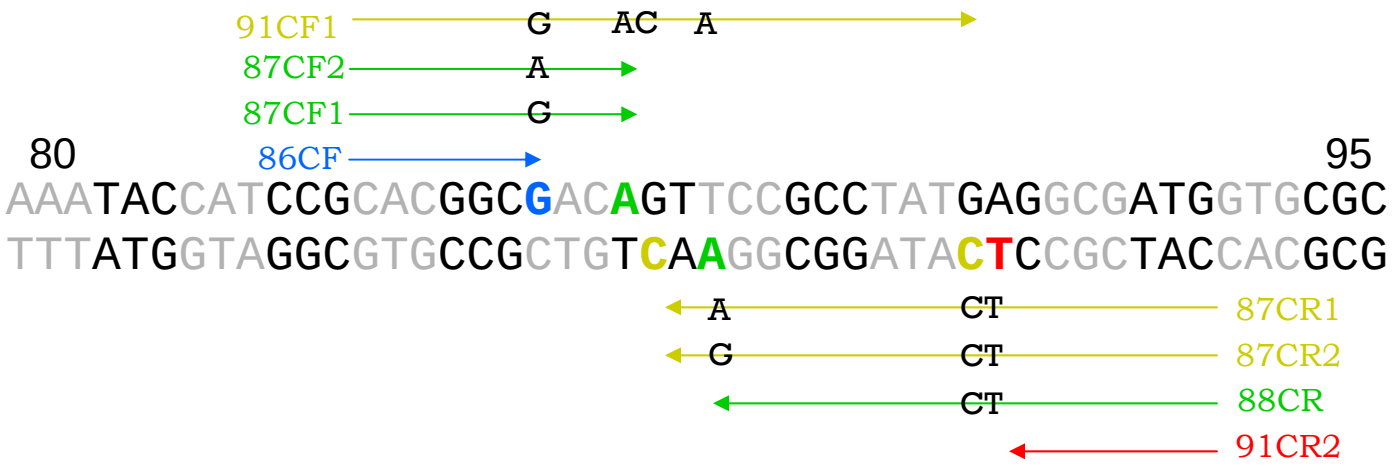


Figure 7: Schematic diagram showing the design of oligonucleotides for *mtr* SNPs using wild type strain FA1090 and a mutated strain. A) 163 bp sequences of *mtr* with SNPs highlighted in yellow. Sequences highlighted in grey are blown up in panel B as double stranded DNA. B) Two oligonucleotides were designed to identify a deletion and a dinucleotide insertion. Oligonucleotide MA was designed on the forward strand to identify an A nucleotide (green) in wild type DNA and a G nucleotide (blue) in DNA containing the A deletion. Oligonucleotide MT was designed on the reverse strand to identify a G nucleotide in wild type DNA and an A nucleotide in DNA containing a dinucleotide TT insertion.

A

mtr

FA1090 –Wild Type

GCAGTCTCAATTTTATGGGTTTCATTATACATACACGATTGCACGGATAAAAAGTCTTTTTTATAATCCGCCCTCGTCAAACCGACCCGAAACGAAAACGCCATTAT
GAGAAAAACCAAAACCGAAGCCTTGAAAACCAAAGAACACCTGATGCTTGCCGCCT

Mutated Strain

GCAGTCTCAATTTTATGGGTTTCATTATACATACACGATTGCACGGATAAAAGTCTTTTTTTTATAATCCGCCCTCGTCAAACCGACCCGAAACGAAAACGCCATTA
TGAGAAAAACCAAAACCGAAGCCTTGAAAACCAAAGAACACCTGATGCTTGCCGCCT

B

FA1090 –Wild Type

Figure 8: Schematic diagram showing the design of oligonucleotides for *norM* SNPs using wild type strain FA1090 DNA. A) 582 bp sequence of *norM* with SNPs highlighted in yellow. Sequences highlighted in blue and grey are blown up in panel B as double stranded DNA. B) The highlighted blue sequence depicts a SNP within the –35 promoter region. Varying lengths of oligonucleotide 35N was designed on both the forward and reverse strands with the arrow tip ending on the SNP. The highlighted grey sequence depicts a novel SNP and a SNP within the ribosomal binding site. Oligonucleotide norM was designed on the forward strand with the arrow tip ending on the novel SNP (deleted in mutated strains). Oligonucleotide 7N was designed with varying lengths on both the forward and reverse strands with the arrow tip ending on the RBS SNP. Blue arrows identify guanosine (G) residues, yellow arrows identify cytosine (C) residues, green arrows identify adenosine (A) residues, and red arrows identify thymidine (T) residues.

.

A

norM

AAACGGCGATGCTTCCTTCCGATTCAGAATCCGAACAGCCCCTGTCCGACGGCTCAATGCCGTCAAGTCGTTAAAACCAAACTTTACCATAAAATACACACAATCTG
AATATACTTTATATACCCCGTATCCGCCGTCTGACGGCACGGTATTTTTTACTATATAATGTGCACCATTATTTCTCATCACTTCCCGCTGCCGTTTTTTTTGTTTC
AGACGGCATTTTTATTGACTGAACACTTATGCTGCTCGACCTCGACCGCTTTTCCTTTTCCGTCTTCCTGAAAGAAATCCGCCTGCTGACCGCCCTTGCCCTGCCC
ATGCTGTTGGCGCAGGTCGCGCAGGTGGGCATCGGTTTCGTCGATACCGTGATGGCGGGCGGTGCGGGCAAGGAAGATTTGGCGGCGGTGGCTTTGGGCAGCAGCGC
GTTTGCCACGGTTTATATTACCTTTATGGGCATTATGGCGGCGCTGAACCCGATGATTGCCCAGCTTTACGGCGCGGGTAAAACCGGTGAAGCAGGCGAAACGGGGC
GGCAGGGGATTTGGTTCGGGCTGATTTTGGGGATTTTCGGCATGATTT

B

2.7 Single SNaPshot reactions

Thirty µL of PCR products were purified by incubating at 37˚C for 1 hour in a 1.5 mL tube containing 10 units Shrimp Alkaline Phosphatase (SAP) (USB Corporation, Cleveland OH) and 0.4 units exonuclease (ExoI) (USB Corporation, Cleveland OH). An additional incubation of 15 minutes at 75˚C was performed to deactivate the enzymes. Next, 3 µL of this purified product was mixed with 2.5 µL of SNaPshot Reaction mix (Applied Biosystems), 3.5 µL of ddH_2O, and 1 µL of the SNaPshot oligonucleotide diluted to 0.6 pmol/µL. This 10 µL mixture was placed in the thermal cycler under the following cycle: 25 cycles of 96˚C for 10 seconds, 50˚C for 5 seconds, and 60˚C for 30 seconds. After the SNaPshot PCR, 1 unit of SAP was added for 60 minutes and incubated at 37˚C followed by a 15 minute incubation at 75˚C. Prior to loading samples onto the capillary electrophoresis analyzer, 1 µL of the sample mixture was added to 9 µL of denaturing Hi-di formamide and 0.1 µL of Genescan LIZ 120 Standard (Applied Biosystems). This sample was heated at 95˚C for 5 minutes and then cooled on ice for 5 minutes before loading to denature the DNA into single strands for electrophoresis.

2.8 Multiplex SNaPshot reactions

Multiplex reactions were performed similarly to single reactions with a few modifications. One µL of each of the desired SNaPshot oligonucleotides was added to 2.5 µL SNaPshot multiplex PCR mix along with 3 µL purified DNA. The samples were purified by dialysis to remove mineral ions from competing with negatively charged DNA for entry into the capillary during electrophoresis (17). Following the

second SAP purification, the products were placed on a filter membrane shiny side up (0.025 µm pore size, VSWP 02500, Millipore, Bedford, MA) floated on 50 mL of deionized, autoclaved water in a 50 mL conical tube for 20 minutes. The samples were then carefully pipetted off and diluted in Hi-di formamide before loading as described for single reactions.

2.9 SNaPshot Primer Focus

It can often be difficult to predict the mobility of oligonucleotides since their final mobility is determined not only by their length, but also by the molecular weight of the labelling dye. The SNaPshot Primer Focus Kit (Applied Biosystems) was designed to screen oligonucleotides for approximate size locations prior to multiplexing to minimize the possibility of multiplexing overlapping products. This way a preview of all potential products can be determined by calculating the mobility shift for each allele. Similarly to the SNaPshot reaction, a single base extension reaction adds fluorescently labeled ddNTPs to the oligonucleotide of interest but without the presence of a DNA template. The result is an electropherogram with four unique peaks that each corresponds to a specific nucleotide, A (green), C (black), G (blue) or T (red). A master mix functional for analyzing up to ten oligonucleotides was prepared, consisting of 40 µL SNaPshot Primer Focus ddNTP mix, 10µL SNaPshot Primer Focus 10x Reaction Buffer, 10 µL SNaPshot Primer Focus Cofactor, 1 µL SNaPshot Primer Focus Enzyme and 29 µL of deionized autoclaved water. The master mix was aliquoted into 9 µL equivalents. In the first equivalent, 1 µL of a 2 µM SNaPshot Primer Focus Control was added. To the

remaining equivalents 1 µL of the desired oligonucleotide at a concentration of 0.6 pmol was added. Samples were placed in the thermocycler at 37˚C for 15 minutes followed at 70˚C for 10 minutes. Purification and further processing of samples was identical to single SNaPshot reactions after the SNaPshot PCR.

2.10 Capillary Gel Electrophoresis

SNP detection was carried out on a capillary PRISM 310 automated sequencer (Applied Biosystems, Foster City, CA). The capillary utilized in this study was from Applied Biosystems and had dimensions of 47 cm x 50 µm. Performance optimized polymer-4 (POP-4) was injected into the capillary to serve as a dynamic coating for the capillary walls and to provide a sieving medium for ssDNA separation (17). The electrophoretic buffer used was 1x Genetic Analyzer buffer with EDTA (diluted from 10x, Applied Biosystems). Electrophoresis parameters were set at 15.0 kV for an injection voltage and 15.0 kV for a run voltage, with a run temperature of 60˚C and run time of 24 minutes. An injection time of 10 seconds was determined optimal for producing sufficiently intense fluorescent peaks while minimizing artifact peaks. Samples were analyzed using Genescan Analysis Software version 3.7.

2.11 Urine Collection

Since there is no acceptable animal model (49) and urethral/vaginal swabs from infected persons were not available for this study, we chose to spike normal urine samples with laboratory isolates to imitate natural infections. Urethral scrapings from men with gonorrhea found that gonococci could adhere to epithelial cells of infected mucosa (120). Furthermore, there is an association in infected

exudates with gonococci in epithelial and mucous-secreting cells in that urethral epithelial cells are invaded by *N. gonorrhoeae* during infection (91). Confocal microscopy showed that intracellular gonococci were either in vacuoles or free within the cytoplasm of urethral epithelial cells while acridine orange staining proved that the majority of organisms were viable (49). Many studies have also demonstrated that gonococcal pili are essential for attachment and entry into urethral epithelial cells (77). Thus the four stages of infection can be summarized by attachment of the plasma membrane with the bacterial membrane, uptake of the organism by epithelial cells followed by their uptake into vacuoles, replication within these vacuoles and then the rupture of organisms from the cells into the lumen and infected exfoliated epithelial cells are shed in the urine (49). Gonococci that are released either reinfect other epithelial cells or are excreted into the urine. Therefore, it is important to be able to determine if SNPs from *N. gonorrhoeae* can be detected from within these epithelial cells and also within free urine.

Exfoliated urethral epithelial cells were obtained from 5 freshly voided urine specimens (usually 300 mL) over a 24 hr period from 3 male volunteers without history or evidence of urogenital tract disease. Volunteers were told to void the first 50 mL and to collect the urine sample at midstream. At the time of collection, none of the volunteers were on any antibiotics and were told to abstain from consuming alcoholic products for the 24 hr period prior to sampling and during the experiment itself. Specimens were collected in sterile Nalgene screw top containers (Nalge Company, Rochester, NY) and placed at 4 C immediately after collection. When all

samples had been gathered, 25 mL from each container was pooled into a working flask. The remaining urine was frozen at -80 C.

2.12 DNA isolation in urine processing

DNA was isolated from spiked urine samples using several bacterial species. The following species were studied: *N. gonorrhoeae, E. coli, L. lactococcus, L. acidophilus, M. osloensis* and *S. aureus*. A loopful of cells from each species was collected from a 24 hr culture using the growth requirements described in section 2.1 Bacterial Strains and Conditions, for each respective bacterial species and diluted in diluent buffer (10% salts solution supplemented with 0.54% KH_2PO_4 and 0.05% Bovine Serum Albumin, pH 6.9) to a concentration of greater than 10^9 cells/mL. This suspension was then diluted 1:10 with pooled urine in a flask, unshaken and incubated at 37 C at 7% CO_2 to simulate an *in vivo* scenario. At 1 hr, 4 hr, and 24 hr intervals, 1 mL duplicate samples in microfuge tubes were taken of the urine cultures and spun in a microfuge (Eppendorf Centrifuge 5417C, Mississauga, ON) at 8000 x g for 10 mins. The supernatants were pipetted off and DNA from the resulting pellets was isolated in two different ways. Using the first method, the pellet was resuspended in 200 µL of autoclaved, deionized water and heated to 95 C for 10 mins. The second method involved the use of a DNA isolation kit for extracting DNA from clinical samples (including urine) (MagNA Pure LC DNA Isolation Kit III (Bacteria, Fungi), Roche Applied Science, Penzberg, Germany). In brief, the pellet was resuspended well in 130 µL of a bacterial lysis buffer mix and then 20 µL of a reconstituted proteinase K solution was added and incubated at 65 C for 10 mins.

Finally, the sample was heated at 95 C for 10 mins to breakdown excess protein. DNA from both methods was amplified using PCR with *gyrA* and *parC* primers (Table 4). Amplicons (10 µL) were run on an agarose gel to determine if amplified product could be visualized. Amplicons were also processed for SNP detection using capillary electrophoresis with multiplexed *gyrA* and *parC* oligonucleotides (Table 5) to determine if SNPs could be identified from clinical urine samples.

Additionally, the lower limits of SNP detection were examined using the *gyrA* oligonucleotide 95AF1 and the *parC* oligonucleotide 87CR1 for *N. gonorrhoeae*. For this experiment, an 18 hr culture of *N. gonorrhoeae* strain 19703 was diluted to a McFarland standard of 0.5 or approximately 10^8 cells/mL. Cell suspensions were diluted to 10^7, 10^6, 10^5, 10^4, 10^3, and 10^2 cells/mL and DNA was quantitatively extracted from 1 mL samples similarly to the aforementioned protocol at 1, 4, and 24 hr increments to determine the minimal requirements for SNP detection.

3. RESULTS

3.1 Determining efflux function differences between two *N. gonorrhoeae* strains using sequencing, intracellular accumulation and transformation studies.

From previous work by Ng *et al*., it was found that two *N. gonorrhoeae* strains 19703 and 21146 differed in their ciprofloxacin MIC values but no difference in their *gyrA* and *parC* QRDRs mutations were noted (Table 3) (86). Mutations within these regions of DNA are typical of high-level ciprofloxacin resistance (72). Since these two strains shared the same *gyrA* and *parC* mutation profiles, this led us to believe that a possible novel efflux transporter could be the cause of the 2-fold increase in MIC in 19703. In 1998, Morita *et al*. discovered the NorM efflux system in *V. parahaemolyticus* that could pump out the fluoroquinolones ciprofloxacin and norfloxacin (82). We aligned DNA sequences of conserved regions of this pump using the *N. gonorrhoeae* genome (www.genome.ou.edu) to determine if a homologous system may exist. It was discovered that the conserved GKFGXP protein sequence belonging to *V. parahaemolyticus* was also present in *N. gonorrhoeae* within the gene NG0395 of the FA1090 gonococcal genome (data not shown). This was later confirmed in February 2003 by Rouquette-Loughlin *et al*. Their group found two mutations, one within a –35 putative promoter region (CTGACG) and one within a likely ribosomal binding site 7 bp upstream of the *norM* ATG start site (TGAA), that resulted in increased resistance to ciprofloxacin and

norfloxacin (94). In order to determine if similar mutations existed within the *norM* gene of our strains, primers were designed (Table 4) to amplify a 582 bp region of the *norM* gene with promoter that were sequenced. A 180 bp region of this sequence is shown in Figure 9. Besides 19703 and 21146 several other strains were examined for *norM* mutations, including two other ciprofloxacin resistant strains (18097,19081), four strains having *mtr* deletions (19491, 21631, 24396, 24425) and FA1090 for comparison to the sequence NG0395. The *mtr* strains were used for comparison since they are not capable of effluxing ciprofloxacin and therefore should not be affected by the addition of CCCP. A *N. gonorrhoeae* strain with the –35 putative promoter and RBS was acquired from Rouquette-Laughlin *et al.* (*gyrA* mutations S91F, D95G, *parC* mutations S88P E91K, ciprofloxacin MIC 4 µg/mL) and sequenced to verify the location of the mutations and provide a comparison to the other strains tested. Remarkably none of the strains including 19703 reported the –35/RBS mutations. However, all of the *mtr* deletion strains and three of the ciprofloxacin resistant strains shared a common T deletion in a region flanked by the –35 and RBS mutations. Comparing these strains to their ciprofloxacin MICs (Appendix A) all were resistant to ciprofloxacin (MIC ≥ 1µg/mL) except 21631, 24396 and 24425.

Figure 9: Sequencing analysis for a portion of the *norM* promoter region with several *N. gonorrhoeae* strains. *NorM* primers were used from Table 4. Sequences were compared to NG0395, the genomic sequence for *norM* in strain FA1090 (www.stdgen.lanl.gov/stdgen/bacteria/ngon/index). FA1090 was sequenced as a control. 19491, 21631, 24396, and 24425 were strains with an *mtr* deletion. 18907, 19081, and 19703, and 21146 were ciprofloxacin resistant strains. The –35/RBS strain was acquired from Dr. William Shafer, Emory University, Atlanta, GA. Deletions and point mutations are shaded in black. The ATG start site for *norM* is highlighted in grey text. The putative –35 promoter region and the putative ribosomal binding site are also shown. The –10 region has not yet been determined.

```
                                -35
NG0395          TATATACCCCGTATCCGCCGTCTGACGGCACGGTATTTTTTACTATATAATGTGCACCATT 61
FA1090          TATATACCCCGTATCCGCCGTCTGACGGCACGGTATTTTTTACTATATAATGTGCACCATT 61
19491           TATATACCCCGTATCCGCCGTCTGACGGCACGGTATTTTTTACTATATAATGTGCACCATT 61
21631           TATATACCCCGTATCCGCCGTCTGACGGCACGGTATTTTTTACTATATAATGTGCACCATT 61
24396           TATATACCCCGTATCCGCCGTCTGACGGCACGGTATTTTTTACTATATAATGTGCACCATT 61
24425           TATATACCCCGTATCCGCCGTCTGACGGCACGGTATTTTTTACTATATAATGTGCACCATT 61
18907           TATATACCCCGTATCCGCCGTCTGACGGCACGGTATTTTTTACTATATAATGTGCACCATT 61
19081           TATATACCCCGTATCCGCCGTCTGACGGCACGGTATTTTTTACTATATAATGTGCACCATT 61
19703           TATATACCCCGTATCCGCCGTCTGACGGCACGGTATTTTTTACTATATAATGTGCACCATT 61
21146           TATATACCCCGTATCCGCCGTCTGACGGCACGGTATTTTTTACTATATAATGTGCACCATT 61
-35/RBS         TATATACCCCGTATCCGCCGTTTGACGGCACGGTATTTTTTACTATATAATGTGCACCATT 61
                                                                      RBS
NG0395          ATTTCTCATCACTTCCCGCTGCCGTTTTTTTTGTTTCAGACGGCATTTTTATTGACTGAAC 122
FA1090          ATTTCTCATCACTTCCCGCTGCCGTTTTTTTTGTTTCAGACGGCATTTTTATTGACTGAAC 122
19491           ATTTCTCATCACTTCCCGCTGCCGTTTTTTT-GTTTCAGACGGCATTTTTATTGACTGAAC 121
21631           ATTTCTCATCACTTCCCGCTGCCGTTTTTTT-GTTTCAGACGGCATTTTTATTGACTGAAC 121
24396           ATTTCTCATCACTTCCCGCTGCCGTTTTTTT-GTTTCAGACGGCATTTTTATTGACTGAAC 121
24425           ATTTCTCATCACTTCCCGCTGCCGTTTTTTT-GTTTCAGACGGCATTTTTATTGACTGAAC 121
18907           ATTTCTCATCACTTCCCGCTGCCGTTTTTTTTGTTTCAGACGGCATTTTTATTGACTGAAC 122
19081           ATTTCTCATCACTTCCCGCTGCCGTTTTTTT-GTTTCAGACGGCATTTTTATTGACTGAAC 121
19703           ATTTCTCATCACTTCCCGCTGCCGTTTTTTT-GTTTCAGACGGCATTTTTATTGACTGAAC 121
21146           ATTTCTCATCACTTCCCGCTGCCGTTTTTTT-GTTTCAGACGGCATTTTTATTGACTGAAC 121
-35/RBS         ATTTCTCATCACTTCCCGCTGCCGTTTTTTTTGTTTCAGACGGCATTTTTATTGACTGGAC 122

NG0395          ACTTATGCTGCTCGACCTCGACCGCTTTTCCTTTTCCGTCTTCCTGAAAGAAATCCGC 180
FA1090          ACTTATGCTGCTCGACCTCGACCGCTTTTCCTTTTCCGTCTTCCTGAAAGAAATCCGC 180
19491           ACTTATGCTGCTCGACCTCGACCGCTTTTCCTTTTCCGTCTTCCTGAAAGAAATCCGC 179
21631           ACTTATGCTGCTCGACCTCGACCGCTTTTCCTTTTCCGTCTTCCTGAAAGAAATCCGC 179
24396           ACTTATGCTGCTCGACCTCGACCGCTTTTCCTTTTCCGTCTTCCTGAAAGAAATCCGC 179
24425           ACTTATGCTGCTCGACCTCGACCGCTTTTCCTTTTCCGTCTTCCTGAAAGAAATCCGC 179
18907           ACTTATGCTGCTCGACCTCGACCGCTTTTCCTTTTCCGTCTTCCTGAAAGAAATCCGC 180
19081           ACTTATGCTGCTCGACCTCGACCGCTTTTCCTTTTCCGTCTTCCTGAAAGAAATCCGC 179
19703           ACTTATGCTGCTCGACCTCGACCGCTTTTCCTTTTCCGTCTTCCTGAAAGAAATCCGC 179
21146           ACTTATGCTGCTCGACCTCGACCGCTTTTCCTTTTCCGTCTTCCTGAAAGAAATCCGC 179
-35/RBS         ACTTATGCTGCTCGACCTCGACCGCTTTTCCTTTTCCGTCTTCCTGAAAGAAATCCGC 180
```

Most studies working with *norM* homologues in other bacterial species, NorM in *V. parahaemolyticus* (83), HmrM in *Haemophilus influenzae* (124), BexA in *Bacteroides thetaiotaomicron* (79) and YdhE in *E. coli* (125), have shown that these efflux pumps use energy via the proton motive force (PMF) similar to other MATE pumps. Therefore, the PMF uncoupler CCCP was used to determine the functionality of the NorM pump by measuring differences in ciprofloxacin MICs. As CCCP dissipates the PMF, not enough energy can be obtained to efflux the drug, hence lowering the amount of effective drug to kill the cell, and in effect lowering the MIC (128). The MICs of 32 strains were determined using CCCP (Table 6). First however, optimal growing conditions were established as controls. *N. gonorrhoeae* FA1090 was grown on normal GCMB medium and GCMB containing CCCP at concentrations of 6.25, 12.5, 25, and 50 μM with no inhibition in confluent growth observed. In addition, ciprofloxacin MICs were performed on GCMB, on GCMB with acetone, and on GCMB with various concentrations of CCCP (Table 6). The acetone controls were added to ensure proper cell growth since toxicity was a concern as the CCCP was dissolved in acetone. Control strains were used to validate the MIC procedure. Strains with *mtr* deletions were used as negative controls because the addition of CCCP should have had no effect on ciprofloxacin resistance. The remaining strains used were ciprofloxacin resistant with various *gyrA* and *parC* mutations. When MICs were performed with CCCP concentrations of 25 μM and 2.5 μM no growth was observed. When CCCP was diluted to 0.25 μM and 0.5 μM growth was observed but there was little difference between these MICs and standard ciprofloxacin MICs for all of the ciprofloxacin resistant strains. In fact,

there was only one significant difference in MIC in control strain CDC 10328 from an MIC of 2 µg/mL to an MIC of 0.25 µg/mL with 0.5 µM CCCP.

Table 6: Effects of CCCP on MICs of ciprofloxacin resistant *N. gonorrhoeae* strains.

Strain	Description	Ciprofloxacin MIC (µg/mL)	CIP + acetone MIC (µg/mL)	CIP + 0.25µM CCCP MIC (µg/mL)	CIP + 0.5µM CCCP MIC (µg/mL)
I-20	Control strain	0.002	0.004	0.002	0.002
I-21	Control strain	0.004	0.004	0.002	0.002
I-22	Control strain	0.002	0.002	0.002	0.002
ATCC49226	Control strain	0.002	0.004	0.002	0.002
SPL4	Control strain	16	16	8	16
CDC10328	Control strain	2	0.5	0.5	0.25
CDC10329	Control strain	2	1	1	2
FA1090	Reference	0.004	0.008	0.004	0.002
19255	*mtr* deletion	0.008	0.016	0.016	0.008
19283	*mtr* deletion	0.016	0.016	0.008	0.008
19378	*mtr* deletion	0.016	0.008	0.008	0.008
19416	*mtr* deletion	0.016	0.016	0.016	0.016
19491	*mtr* deletion	16	8	8	16
21631	*mtr* deletion	0.002	0.002	0.002	0.002
18610	CIP resistant	16	8	8	8
18907	CIP resistant	16	16	16	16
19081	CIP resistant	1	1	1	1
19132	CIP resistant	8	16	8	8
19280	CIP resistant	16	16	16	16
19286	CIP resistant	8	8	4	8
19289	CIP resistant	8	8	8	8
19316	CIP resistant	1	1	0.5	0.5
19324	CIP resistant	16	16	16	16
19328	CIP resistant	32	16	16	32
19330	CIP resistant	8	4	4	8
19333	CIP resistant	8	8	4	8
19703	CIP resistant	16	16	8	16
19707	CIP resistant	16	16	8	16
20419	CIP resistant	2	2	2	2
20423	CIP resistant	8	8	4	8
21145	CIP resistant	8	4	4	4
21146	CIP resistant	4	4	2	2

CIP – ciprofloxacin, MIC, minimal inhibitory concentration, and CCCP –carbonyl cyanide m-chlorophenyl hydrazone

To further examine the novel *norM*, T deletion detected during sequencing, transformational studies were performed using DNA from strains 19703 and 21146 as donors and strain FA1090 as the recipient strain (Table 7). The resulting constructs had MICs ranging from 0.008 to 0.125 or a 2-fold to a 16-fold difference. The *norM* region of the genome was sequenced for each transformed strain (data not shown). None of these strains showed the T deletion despite the increases in MIC. It was thought that perhaps other genomic mutations had developed in other known fluoroquinolone resistance causing regions due to the selective pressure put on the cells during transformation. Thus, we sequenced the *gyrA* and *parC* QRDRs for these strains (data not shown). No mutations were found in these regions. A 2286 bp region of the *gyrA* gene was also sequenced using primers gyrA-F2 through to gyrA-R7 (Table 4) to see if there were mutations located outside the QRDR (data not shown). Again, no additional mutations were found outside this region.

Table 7: Transformational effects of donor strains DNA on recipient strain FA1090 with resulting transformation MIC to ciprofloxacin.

Transformants Designation	Donor		Recipient		Type of Transformation	Resulting MIC µg/mL
	Strain	MIC µg/mL	Strain	MIC µg/mL		
A	21146	4	FA1090	0.004	Uptake sequence	0.064
B	19703	16	FA1090	0.004	Uptake sequence	0.008
C	19703	16	FA1090	0.004	Uptake sequence	0.008
D	21146	4	FA1090	0.004	Uptake sequence	0.064
E	19703	4	FA1090	0.004	Uptake sequence	0.064
6-19703	19703	4	FA1090	0.004	Uptake sequence	0.125
6-21146	21146	16	FA1090	0.004	Uptake sequence	0.064
7-19703	19703	4	FA1090	0.004	Uptake sequence	0.064
7-21146	21146	16	FA1090	0.004	Uptake sequence	0.125
21146 NorM cip 0.004	21146	16	FA1090	0.004	Liquid	0.064

3.2 Detection of single nucleotide polymorphisms in *N. gonorrhoeae* using single base extension capillary electrophoresis.

3.2.1 Single peak detection of SNPs conferring antibiotic resistance in *gyrA, parC mtr* and *norM* genes

Oligonucleotides used for SNP detection in *gyrA, parC, mtr* and *norM* are described in 2.6 Design of SNaPshot extension primers. Sequences for those primers are shown in Table 5 and Figures 5, 6, 7, and 8 show the positions of these

oligonucleotides with respect to wild type FA1090 DNA. In order to ensure the SNaPshot technology could be applied to identify the desired SNPs, we sought to optimize conditions for single reactions.

In *gyrA*, four oligonucleotides were designed to detect six mutations: three within codon 91 and three within codon 95 (Table 8). Oligonucleotides 95AF1 and 95AF2 were identical except for one nucleotide contained within codon 91 that either coded for a C or T nucleotide. Only one of these oligonucleotides would bind properly to its respective DNA strand. 95AF1 contained a C nucleotide and thus was only able to bind to DNA that contained the wild type genotype at codon 91. 95AF2 contained a T nucleotide and thus was capable of binding to DNA that contained a mutant genotype at codon 91. In strain 19703, oligonucleotide 91AF identified a S91F (TCC→TTC) mutation in the second position of codon 91, 95AR identified a D95G (GAC→GGC) mutation in the second position of codon 95, and 95AF2 identified the wild type G nucleotide (GGC) within the first position of codon 95 while 95AF1 did not bind to the DNA resulting in an absence of a fluorescent signal by capillary electrophoresis (Figure 10). DNA sequencing confirmed these mutations in strain 19703 (data not shown). Additional strains FA1090, 18907, 19081, and 21146 with wild type and mutated nucleotide sequences in *gyrA* codons 91 and 95 were also tested via this method and sequenced to confirm SNP detection (data not shown).

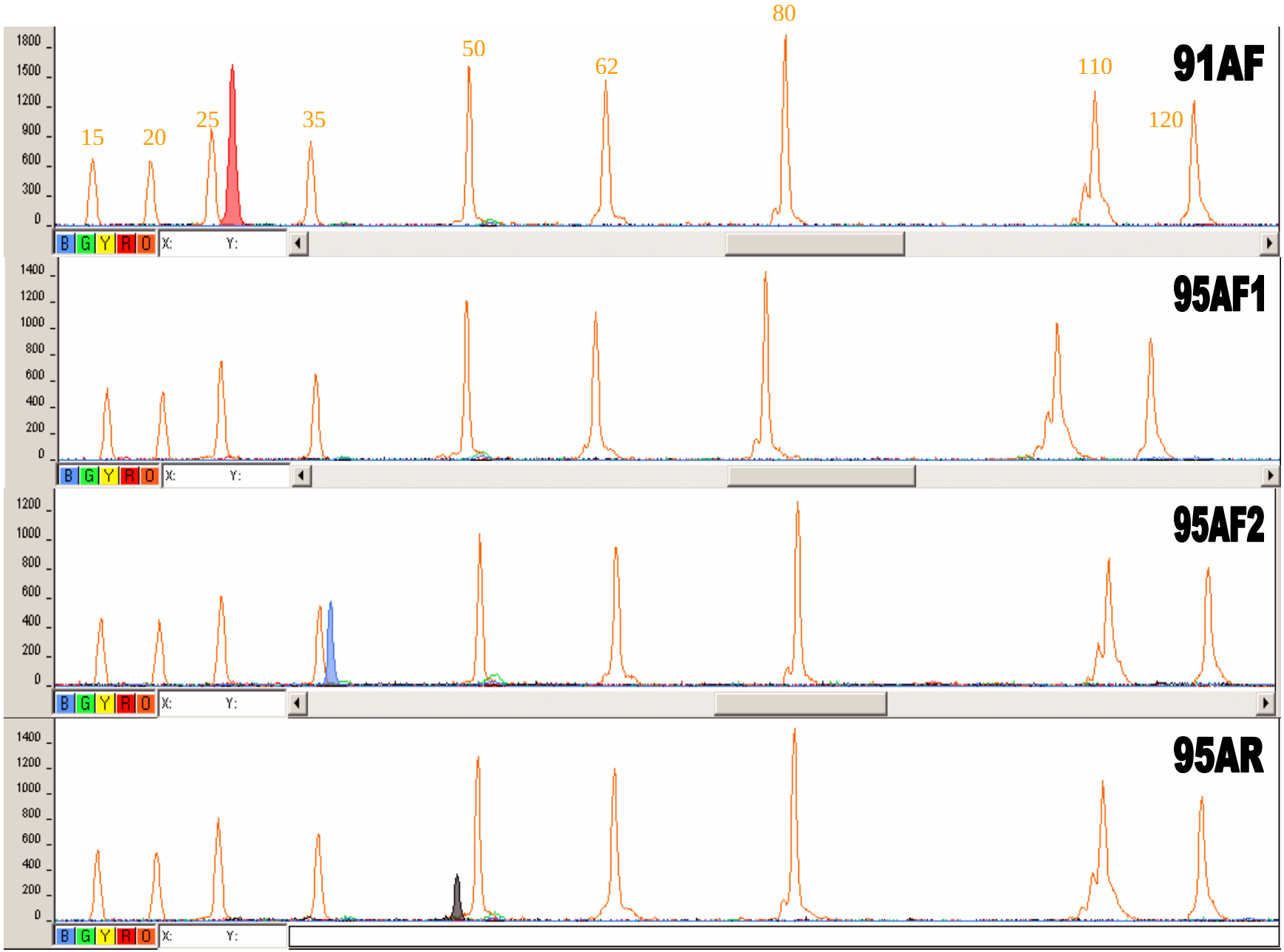
Table 8: Mutations in *gyrA* codons 91 and 95 including the amino acid substitution and nucleotide change.

Mutation Designation	Amino Acid Substitution Nucleotide Change
S91 (wt)[a]	Ser
	TCC
S91F	Ser→Phe
	TCC→TTC
S91Y	Ser→Tyr
	TCC→TAC
S91C	Ser→Cys
	TCC→TGC
D95 (wt)[a]	Asp
	GAC
D95G	Asp→Gly
	GAC→GGC
D95A	Asp→Ala
	GAC→GCC
D95N	Asp→Asn
	GAC→AAC

[a]Indicates wild type (wt) designation at codons 91 and 95.

In *parC*, eight oligonucleotides were designed to detect nine mutations: one within codon 86, three within codon 87, two within codon 88, and three within codon 91 (Table 9). For oligonucleotides that had to be designed overlapping other SNPs, a system was devised to adopt wild type nucleotide designation except where the 3' end of the oligonucleotide was within five nucleotides of the SNP to be identified. In these situations, alternate oligonucleotides were created with both nucleotides in mind (Figure 6). Therefore, oligonucleotides 87CF1 and 87CF2 were identical except for one nucleotide contained within codon 86, three nucleotides adjacent to the SNP, that either coded for an A of G nucleotide. Also, oligonucleotides 87CR1 and 87CR2 were identical except for one.

Figure 10: Electropherograms for single SNPs of *gyrA* for strain 19703. Standard peaks are coloured in orange with their sizes in bp labeled. From the top panel down: oligonucleotide 91AF detected a T nucleotide (red peak) at size 27 bp, 95AF1 detected no SNP, 95AF2 detected a G nucleotide (blue peak) at size 36 bp, and 95AR detected a C nucleotide (black peak) at size 48 bp.


91AF
95AF1
95AF2
95AR
15
20
25
35
50
62
80
110
120
B G Y R O
X:
Y:

nucleotide contained within codon 88, two nucleotides adjacent to the SNP, that either coded for an A or G nucleotide.

For both sets of oligonucleotides only one would bind properly to its respective DNA strand. 87CF1 contained a G nucleotide and thus was only able to bind to DNA that contained the wild type genotype at codon 86. Likewise, 87CR1 contained an A nucleotide and thus was only able to bind to DNA that contained the wild type genotype at codon 88. 87CF2 and 87CR2 contained A and G nucleotides respectively and were capable of binding to DNA that contained a mutant genotype. Otherwise, overlapping oligonucleotides 88CR and 91CF1 and the remaining portions of 87CR1 and 87CR2 were designed with wild type nucleotide designations since any overlapping SNPs were more than five nucleotides away from the 3' ends. In strain 19703, oligonucleotide 86CF identified a wild type G nucleotide (GAC) within the first position of codon 86, 87CF1 identified a S87R (AGT→CGT) mutation within the first position of codon 87, 87CR1 identified a wild type G nucleotide (CGT) within the second position of codon 87, 88CR identified a wild type T nucleotide (TCC) within the first position of codon 88, 91CF1 identified a wild type G nucleotide (GAG) within the first position of codon 91, and 91CR2 identified a wild type A nucleotide (GAG) within the second position of codon 91 (Figure 11). Oligonucleotides 87CF2 and 87CR2 did not bind to the DNA resulting in the absence of a fluorescent signal by capillary electrophoresis (Figure 11). DNA sequencing confirmed these mutations in strain 19703 (data not shown). Additional strains FA1090, 18097, 19081, and 21146 with wild type and mutated nucleotide sequences

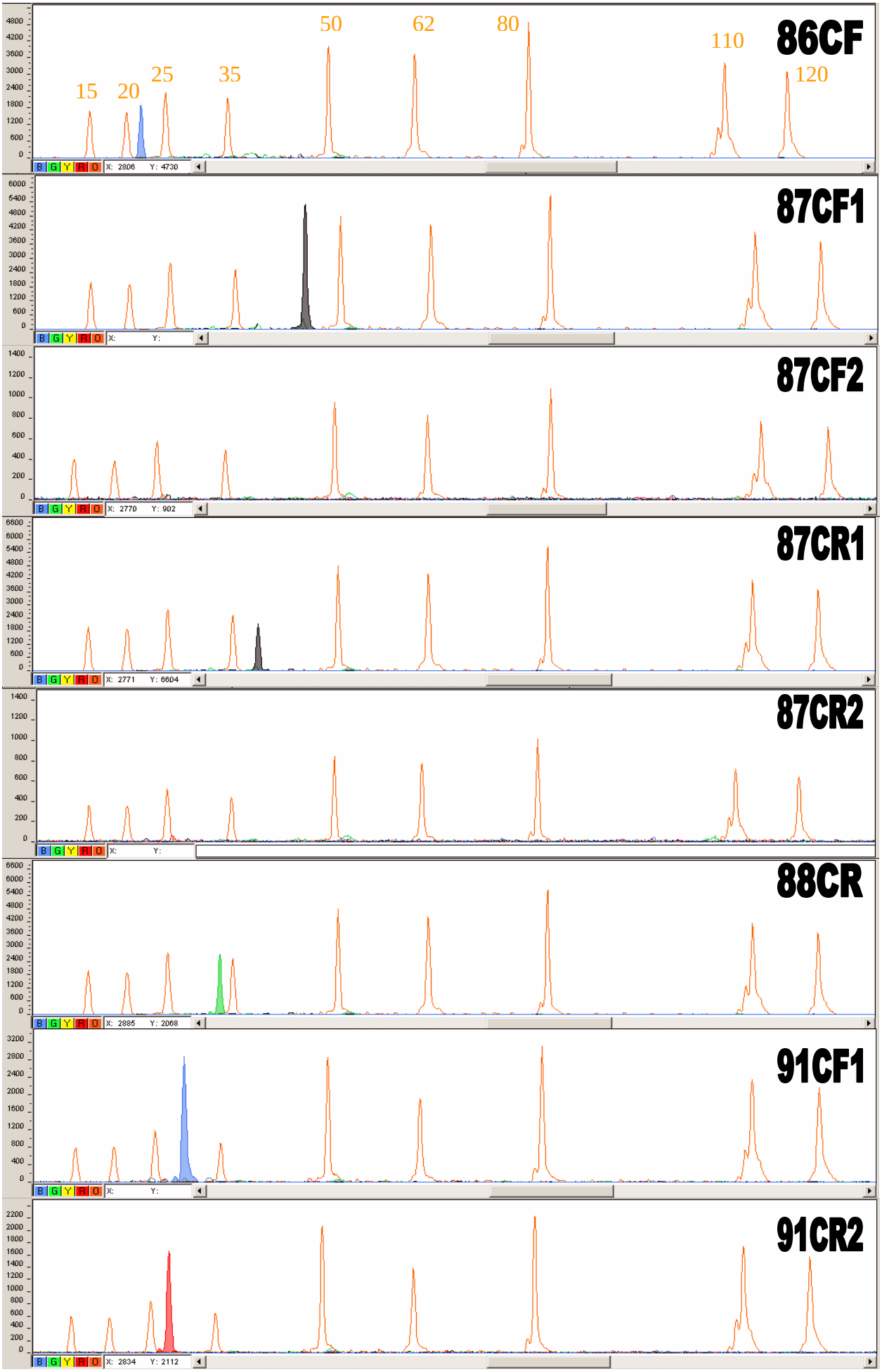
in *parC* codons 86, 87, 88 and 91 were also tested via this method and sequenced to confirm SNP detection (data not shown).

Table 9: Mutations in *parC* codons 86, 87, 88 and 91 including the amino acid substitution and nucleotide change.

Mutation Designation	Amino Acid Substitution Nucleotide Change
D86 (wt)[a]	Asp GAC
D86N	Asp→Asn GAC→AAC
S87 (wt)[a]	Ser AGT
S87I	Ser→Ile AGT→ATT
S87N	Ser→Asn AGT→AAT
887R	Ser→Arg AGT→CGT
S88 (wt)[a]	Ser TCC
S88R	Ser→Arg TCC→CGC
S88P	Ser→Pro TCC→CCC
E91 (wt)[a]	Glu GAG
E91K	Glu→Lys GAG→AAG
E91G	Glu→Gly GAG→GGG
E91Q	Glu→Gln GAG→CAG

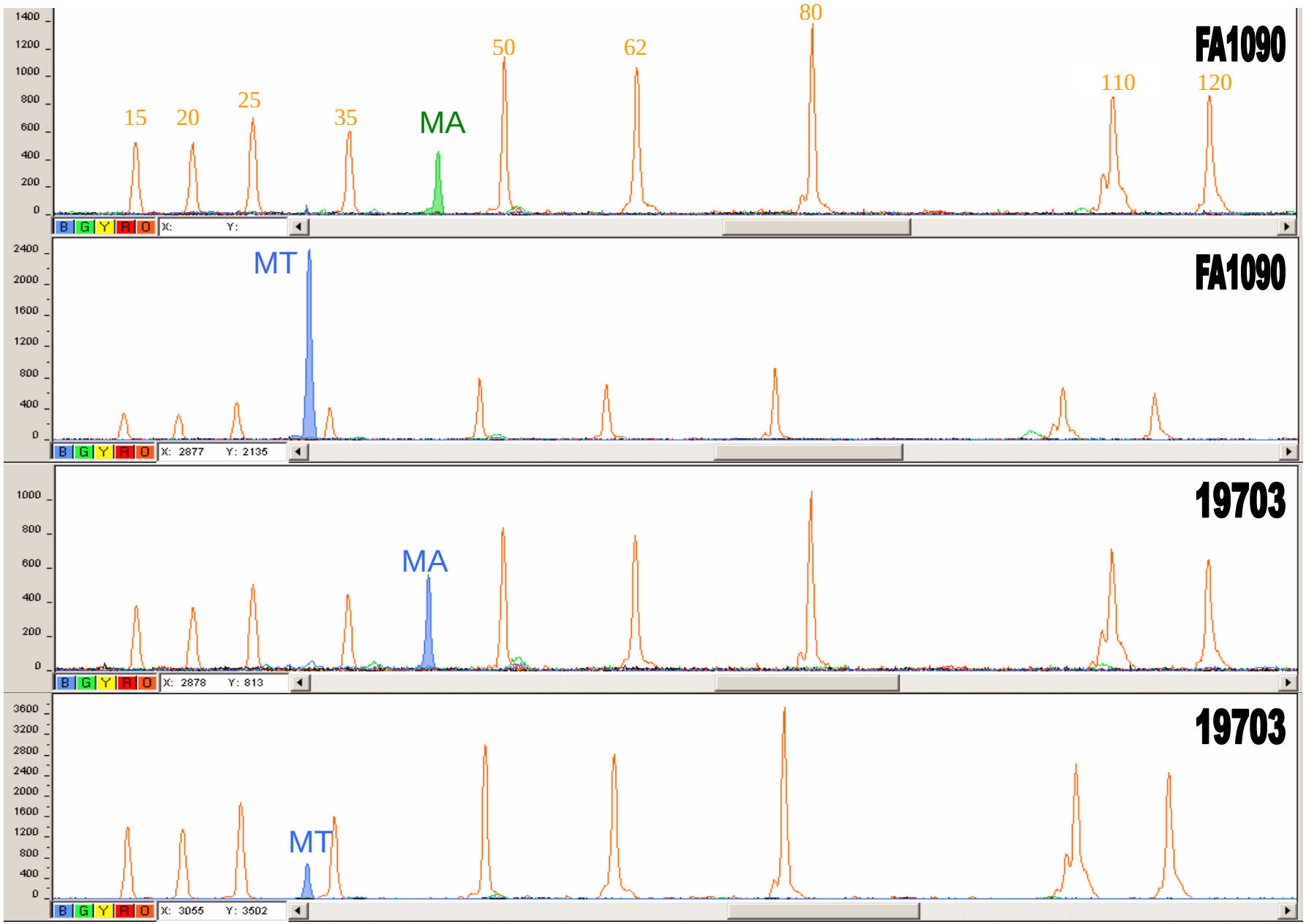
[a]Indicates wild type (wt) designation at codons 86, 87, 88 and 91.

Figure 11: Electropherograms for single SNPs of *parC* for strain 19703. Standard peaks are coloured in orange with their sizes in bp labeled. From the top panel down: oligonucleotide 86CF detected a G nucleotide (blue peak) at size 22 bp, 87CF1 detected a C nucleotide (black peak) at size 45 bp, 87CF2 detected no SNP, 87CR1 detected a C nucleotide (black peak) at size 39 bp, 87CR2 detected no SNP, 88CR detected a A nucleotide (green peak) at size 33 bp, 91CF1 detected a G nucleotide (blue peak) at size 29 bp, and 91CR2 detected a T nucleotide (red peak) at size 28 bp.


86CF
15
20
25
35
50
62
80
110
120
87CF1
87CF2
87CR1
87CR2
88CR
91CF1
91CR2

For *mtr*, two oligonucleotides were designed to detect two mutations: a single deletion and a dinucleotide insertion. Because these mutations are unique in that a nucleotide is either added or deleted instead of substituted, the oligonucleotides were designed differently than for *gyrA* and *parC* SNPs (Figure 7). Instead of the 3' end of the oligonucleotide identifying the SNP, the oligonucleotide was designed over the SNP identifying a different nucleotide if the deletion or dinucleotide insertion is present. Therefore, oligonucleotide MA will always identify a G nucleotide if the single deletion is present, otherwise, an A nucleotide will be detected, indicating a wild type sequence. Similarly, MT will always identify a T nucleotide if the dinucleotide insertion is present, otherwise a C nucleotide will de detected, indicating a wild type sequence. Figure 12 shows *mtrR* SNP detection for strains FA1090 and 19703. In FA1090, MA detected an A nucleotide and MT detected a C nucleotide indicating that FA1090 does not have either of the *mtrR* SNPs. On the other hand, in strain 19703, MA detected a G nucleotide and MT identified a C nucleotide indicating that 19703 contained the single deletion but not the dinucleotide insertion. DNA sequencing confirmed these mutations (data not shown).

Figure 12: Electropherograms for single SNPs of *mtr* for strains FA1090 and 19703. Standard peaks are coloured in orange with their sizes in bp labeled. From the top panel down: In strain FA1090, oligonucleotide MA detected an A nucleotide (green peak) at size 44 bp, and MT detected a G nucleotide (blue peak) at size 33 bp. In strain 19703, MA detected a G nucleotide (blue peak) at size 43 bp, and MT detected a G nucleotide (blue peak) at size 33 bp.


FA1090
15
20
25
35
MA
50
62
80
110
120
FA1090
MT
19703
MA
19703
MT

For *norM*, thirteen oligonucleotides were designed to detect three mutations: one within a –35 putative promoter, one within a likely RBS 7bp upstream of the *norM* ATG start code and the novel T deletion discovered in this study (Figure 8). Five oligonucleotides of varying lengths were designed on both forward and reverse strands for each of the –35 (35N oligonucleotides) and RBS (7N oligonucleotides) mutations without successfully detecting the SNPs (data not shown). Thus, the 35N oligonucleotides could not identify the –35 mutation and the 7N oligonucleotides could not identify the RBS mutation. Three oligonucleotides (NorM, NorM-10, NorM-15) of varying length were designed on the forward strand to detect the novel T deletion at the end of a long run of T residues shown in Figure 8, since the design of an oligonucleotide on the reverse stand would be futile identifying another T in the long series of thymidine residues. Unfortunately, the longer oligonucleotides reporting higher fluorescence signals than the base-line was not comparable to sizing standards (data not shown).

3.2.2 Multiplex detection of SNPs conferring antibiotic resistance in *gyrA, parC* and *mtr*.

Before multiplexing oligonucleotides of each gene (*gyrA, parC*, and *mtr*) into a single reaction, it was necessary to confirm the mobility effects of each potential fluorescent dye being incorporated at the 3' end representing all possible SNPs. This was done in order to predict the size location of the oligonucleotide depending on what SNP was to be identified and to ensure that the resulting peaks of the SNPs would not overlap, making interpretation difficult. Each dye has a different molecular

weight that when added to each oligonucleotide, can cause a mobility shift of that oligonucleotide during electrophoresis (17). The SNaPshot Primer Focus Kit was applied (Section 2.9 SNaPshot Primer Focus) for these purposes. While the oligonucleotides were expected to identify one to four different nucleotides, the Primer Focus Kit attached all four nucleotides individually to the 3' end of the oligonucleotide, thus providing a readout of four peaks representative of each possible SNP. Therefore, in a multiplexed electropherogram it will be possible to accurately label each SNP based on its size location.

In Figure 13, the mobilities of the four *gyrA* oligonucleotides have been shown. Thus, 91AF will identify a SNP between positions 26 to 28, 95AF1 and 95AF2 will identify a SNP between positions 36 to 38 and 95AR will identify a SNP between positions 48 to 49. Note that positions are far enough apart, that when all of the oligonucleotides are multiplexed there will be no chance of peak overlap.

Figure 14, shows the mobilities of the eight *parC* oligonucleotides. Oligonucleotide 86CF will identify a SNP between positions 21 to 28, 87CF1 and 87CF2 will identify SNPs between positions 45 to 46, 87CR1 and 87CR2 will identify SNPs between positions 38 to 40, 88CR will identify a SNP between positions 30 to 33, 91CF1 will identify SNPs between positions 29 to 32 and 91CR2 will identify SNPs between positions 24 to 28. While there is some minor overlap of peaks within 91CF1 and 91CR2 with other SNPs, it is acceptable as long as peaks of the same colour do not overlap.

Figure 15 shows the mobilities of the two *mtr* oligonucleotides. Oligonucleotide MA will identify a SNP between positions 42 and 44, while MT will identify a SNP between positions 33 and 35.

Figure 13: Effect of molecular weight of each fluorescent dye on *gyrA* oligonucleotide mobility during electrophoresis. Standard peaks are coloured in orange with their sizes in bp labeled. From the top panel down: with the extra molecular weight of each dye, 91AF is expected to produce a peak identifying a SNP between sizes 26 to 28 bp, 95AF1 and 95AF2 are expected to produce a peak identifying a SNP between sizes 36 to 38 bp and 95AR is expected to produce peaks identifying a SNP between sizes 48 to 49 bp. Blue peaks identify guanosine (G) residues, yellow peaks identify cytosine (C) residues, green peaks identify adenosine (A) residues, and red peaks identify thymidine (T) residues.

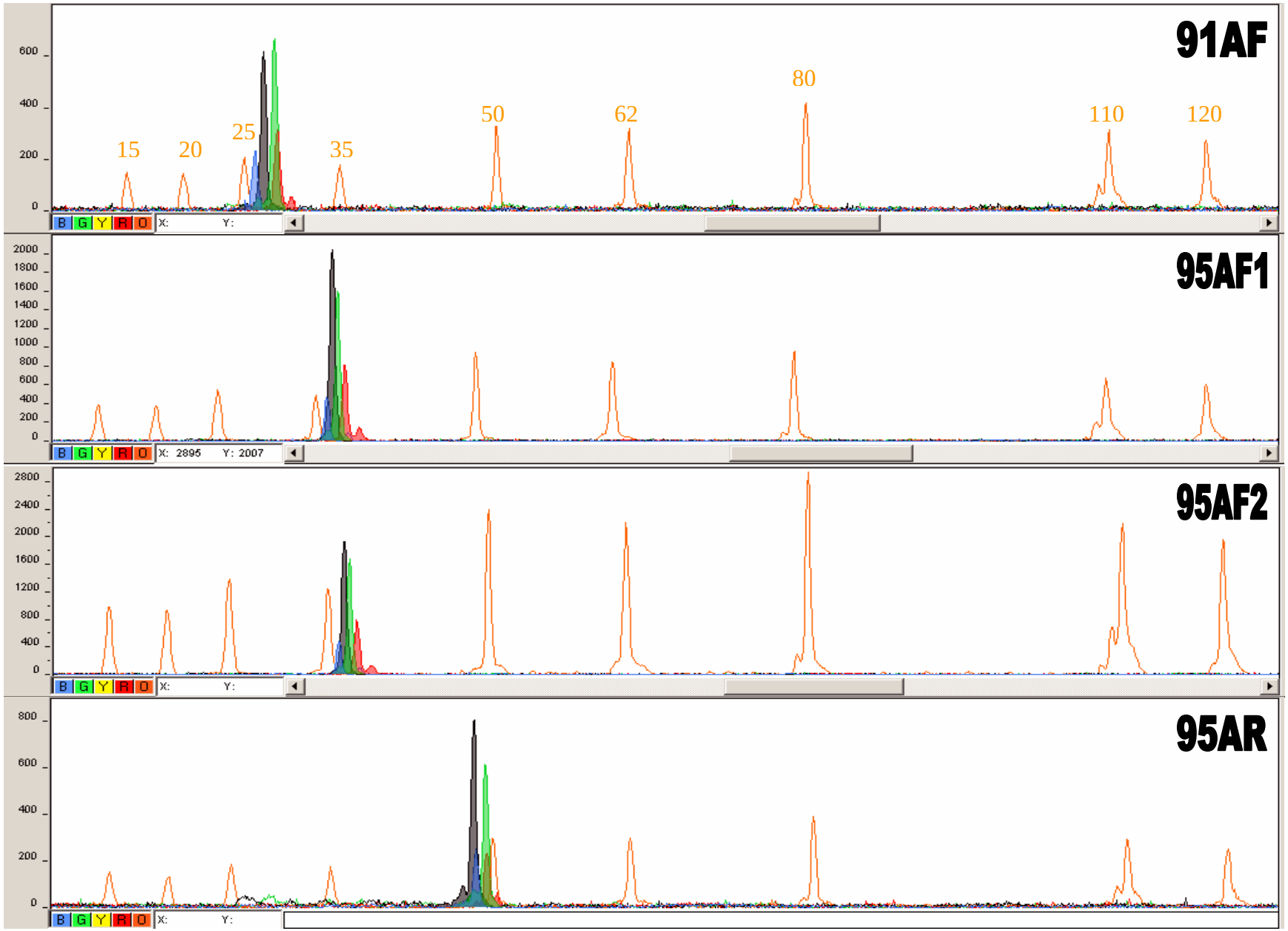

91AF
15
20
25
35
50
62
80
110
120
95AF1
95AF2
95AR
B G Y R O
X:
Y:
X: 2895 Y: 2007

Figure 14: Effect of molecular weight of each fluorescent dye on *parC* oligonucleotide mobility during electrophoresis. Standard peaks are coloured in orange with their sizes in bp labeled. From the top panel down: with the extra molecular weight of each dye, 86CF is expected to produce a peak identifying a SNP between sizes 21 to 28 bp, 87CF1 and 87CF2 are expected to produce peaks identifying a SNP between sizes 45 to 46 bp, 87CR1 and 87CR2 are expected to produce peaks identifying a SNP between sizes 38 to 40 bp, 88CR is expected to produce a peak identifying a SNP between sizes 30 to 33 bp, 91CF1 is expected to produce a peak identifying a SNP between sizes 29 to 32 bp and 91CR2 is expected to produce a peak identifying a SNP between sizes 24 to 28 bp. Blue peaks identify guanosine (G) residues, yellow peaks identify cytosine (C) residues, green peaks identify adenosine (A) residues, and red peaks identify thymidine (T) residues.

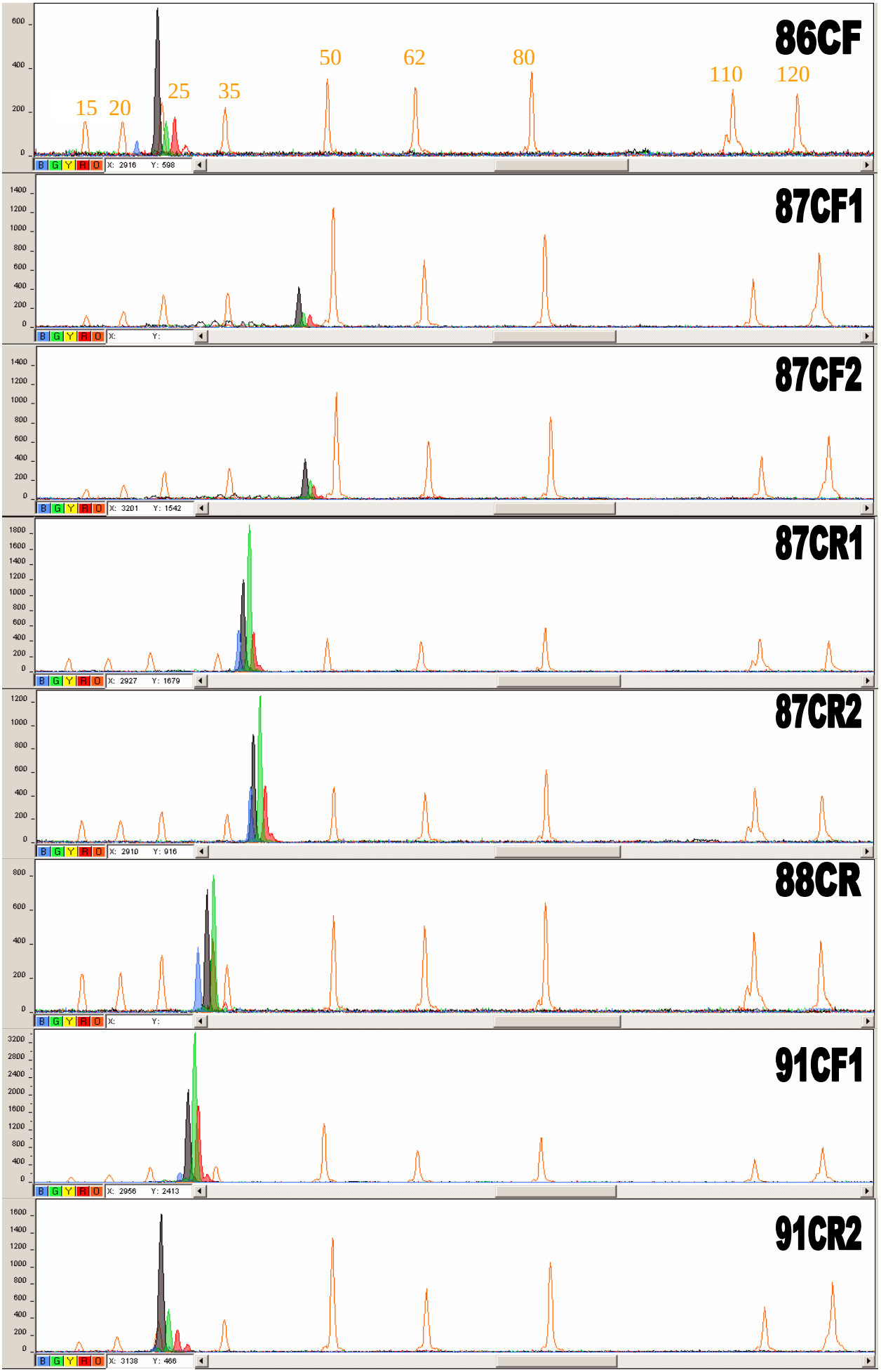
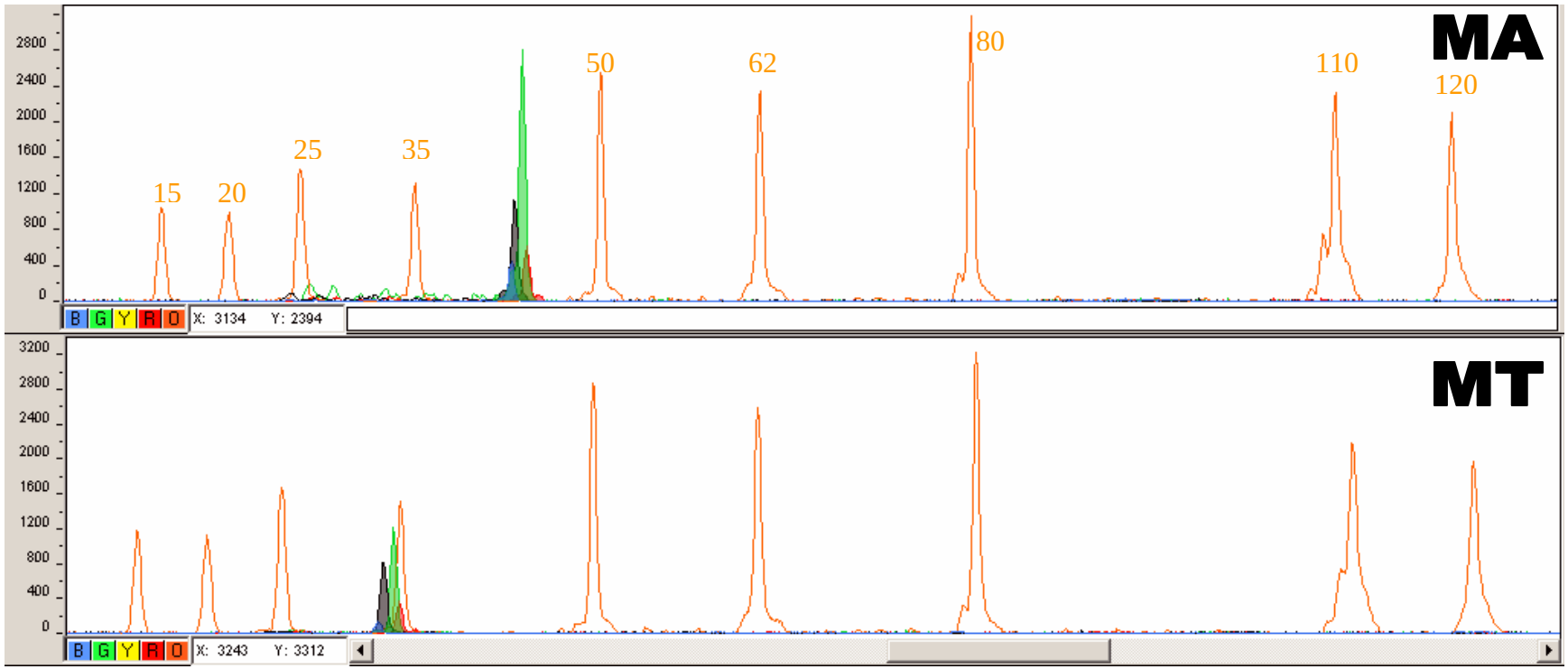

86CF
15
20
25
35
50
62
80
110
120
87CF1
87CF2
87CR1
87CR2
88CR
91CF1
91CR2

Figure 15: Effect of molecular weight of each fluorescent dye on *mtr* oligonucleotide mobility during electrophoresis. Standard peaks are coloured in orange with their sizes in bp labeled. From the top panel down: with the extra molecular weight of each dye, MA is expected to produce a peak identifying a SNP between sizes 42 to 44 bp and MT is expected to produce a peak identifying a SNP between sizes 33 to 35 bp. Blue peaks identify guanosine (G) residues, yellow peaks identify cytosine (C) residues, green peaks identify adenosine (A) residues, and red peaks identify thymidine (T) residues.


MA
15
20
25
35
50
62
80
110
120
2800
2400
2000
1600
1200
800
400
0
B G Y R O
X: 3134 Y: 2394
MT
3200
2800
2400
2000
1600
1200
800
400
0
B G Y R O
X: 3243 Y: 3312

Multiplex electropherograms are shown in Figure 16 for the four *gyrA* oligonucleotides for eight strains: three ciprofloxacin susceptible (MIC < 0.25 µg/mL) (Figure 16, Block A), three intermediate resistant (MIC 0.25-0.5 µg/mL) (Figure 16, Block B) and 2 resistant (MIC ≥ 1 µg/mL) (Figure 16, Block C). For each strain three peaks were observed representing SNPs identified by oligonucleotides 91AF, either 95AF1 or 95AF2 and 95AR, respectively. These strains are represented because they depict all possible SNP combinations observed for *gyrA*. FA1090 in the top panel is a wild type strain with no SNPs, while the remaining strains possess at least one of the SNPs listed in Table 8. Wild type FA1090 shows a black peak in Figure 16, Block A indicating 91AF identifying a C nucleotide was added to the end of this oligonucleotide. Additionally, the blue peak in Figure 16, Block A indicates 95AF1 identifying a G nucleotide was added to the 3' end of this oligonucleotide while a red peak indicates 95AR identifying a T nucleotide. Overall 60 *N. gonorrhoeae* strains were multiplexed with *gyrA* oligonucleotides which included 20 ciprofloxacin susceptible, 20 intermediate resistant, and 20 resistant (Table 10). The 60 strains selected for multiplexing were arbitrarily chosen from the National Microbiology Laboratory STD surveillance data based on their ciprofloxacin MICs. Full antimicrobial susceptibility information for all strains is located in Appendix A. The *gyrA* QRDRs of all 60 strains were sequenced and codons 91 and 95 are displayed in Table 10, verifying the results obtained by capillary electrophoresis. From Table 8, the most common *gyrA* SNPs that were found in this study were S91F (TCC→TTC) in 40 strains (66.7%), D95G (GAC→GGC) in 11 strains (18.3%), D95N (GAC→AAC) in 10 strains (16.7%) and D95A (GAC→GCC) in 6 strains (10%)

(Table 10). Only 1 strain had the S91Y (TCC→TAC) mutation and none contained in S91C (TCC→TGC) mutation (Table 10).

Figure 16: Electropherograms for multiplex SNPs of *gyrA* for multiple strains. Standard peaks are coloured in orange with their sizes in bp labeled. Representative strains from the sensitive, intermediate resistant, and resistant groups show all observable SNP combinations as illustrated in block A, block B and block C respectively. Oligonucleotides 91AF, 95AF1 or 95AF2 and 95AR respectively detected SNPs for each strain whereas each coloured peak corresponds to a specific nucleotide: Black-C, Blue-G, Green-A, and Red-T.

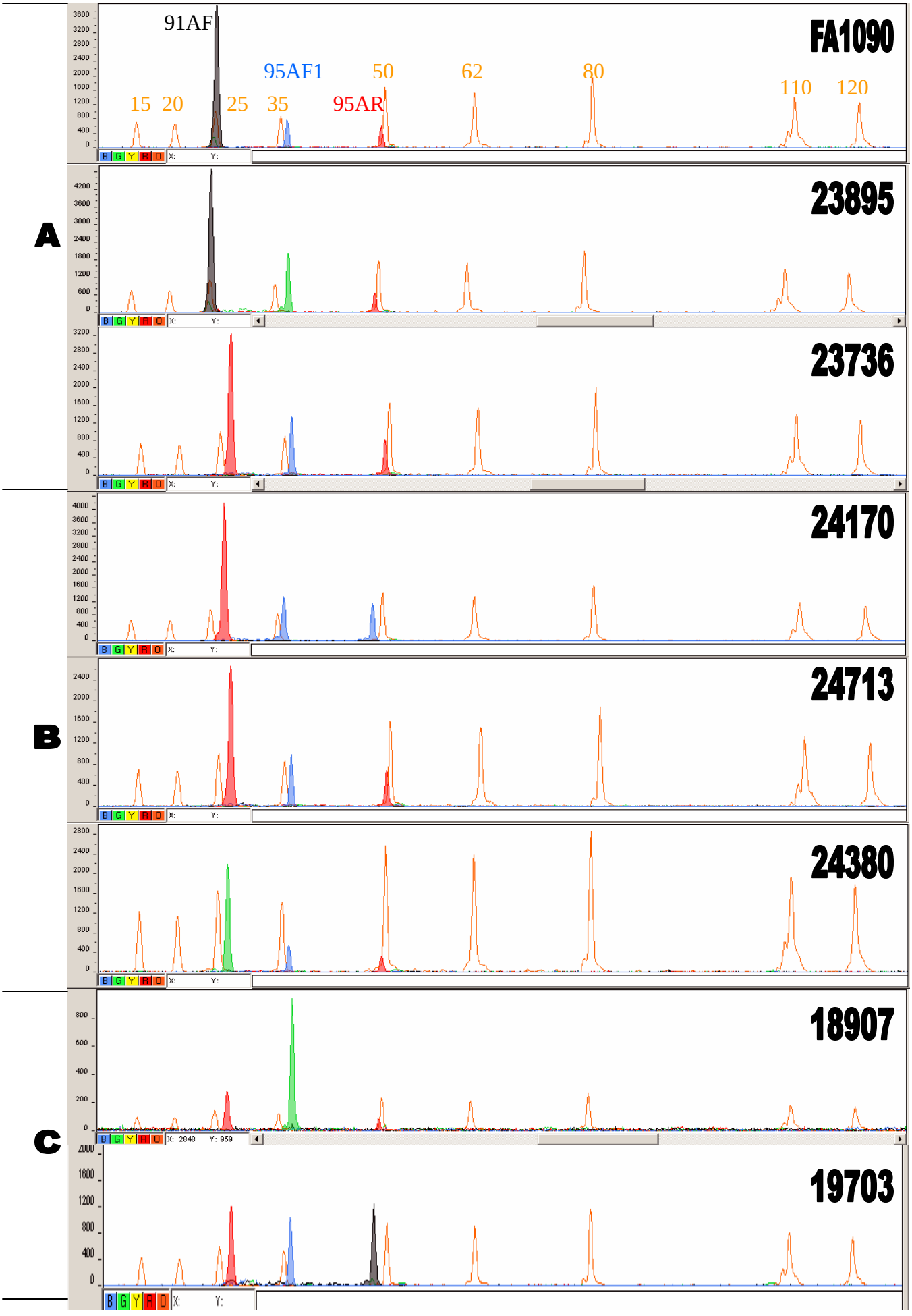

A
B
C
91AF
95AF1
95AR
15
20
25
35
50
62
80
110
120
FA1090
23895
23736
24170
24713
24380
18907
19703

Table 10: *N. gonorrhoeae* strains used including their ciprofloxacin MICs (μg/mL), *gyrA* mutations on codons 91 and 95 and the respective SNPs observed with capillary electrophoresis.

Footnote: Block A represents ciprofloxacin susceptible strains (MIC<0.25 μg/mL), Block B represents ciprofloxacin intermediate resistant strains (MIC 0.25-0.5 μg/mL) and Block C represents ciprofloxacin resistant strains (MIC>1 μg/mL).
[a] SNPs at codons 91 and 95 are highlighted in yellow.
[b] 91AF identified the SNP at the second position of codon 91.
[c] only one of oligonucleotides 95AF1 and 95AF2 was able to identify the SNP at the first position of codon 95.
[d] 95AR identified the SNP at the second position of codon 95 (reverse strand).

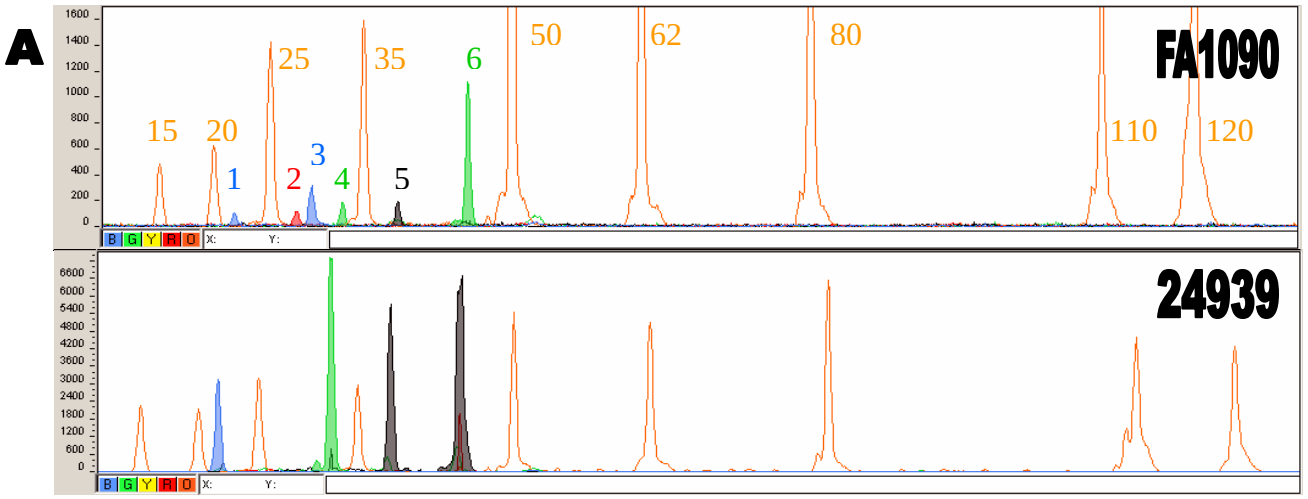
	Strain	MIC	91[a]	95[a]	91AF[b]	95AF1[c]	95AF2[c]	95AF3[d]
A	FA1090	0.004	TCC	GAC	C	G	-	T
	25714	0.004	TCC	GAC	C	G	-	T
	25727	0.004	TCC	GAC	C	G	-	T
	25713	0.008	TCC	GAC	C	G	-	T
	25716	0.008	TCC	GAC	C	G	-	T
	25718	0.008	TCC	GAC	C	G	-	T
	25719	0.008	TCC	GAC	C	G	-	T
	25729	0.016	TCC	GAC	C	G	-	T
	25733	0.016	TCC	GAC	C	G	-	T
	25742	0.016	TCC	GAC	C	G	-	T
	24967	0.032	TCC	GAC	C	G	-	T
	24974	0.032	TCC	GAC	C	G	-	T
	25722	0.032	TCC	GAC	C	G	-	T
	23895	0.064	TCC	AAC	C	A	-	T
	24939	0.064	TTC	GAC	T	-	G	T
	24795	0.064	TCC	GAC	C	G	-	T
	25450	0.125	TTC	GAC	T	-	G	T
	25717	0.125	TTC	GAC	T	-	G	T
	25736	0.125	TTC	GAC	T	-	G	T
	25737	0.125	TTC	GAC	T	-	G	T
B	23937	0.25	TTC	GAC	T	-	G	T
	23953	0.25	TCC	AAC	C	A	-	T
	24058	0.25	TTC	GAC	T	-	G	T
	24059	0.25	TTC	GAC	T	-	G	T
	24118	0.25	TTC	GAC	T	-	G	T
	24146	0.25	TCC	AAC	C	A	-	T
	24170	0.25	TTC	GCC	T	-	G	G
	24178	0.25	TCC	AAC	C	A	-	T
	24179	0.25	TCC	AAC	C	A	-	T
	24713	0.25	TTC	GAC	T	-	G	T
	23536	0.5	TTC	GAC	T	-	G	T
	24078	0.5	TTC	GAC	T	-	G	T
	24364	0.5	TTC	GAC	T	-	G	T
	24380	0.5	TAC	GAC	A	-	G	T
	24437	0.5	TTC	GAC	T	-	G	T
	24489	0.5	TTC	GAC	T	-	G	T
	25092	0.5	TTC	GAC	T	-	G	T
	25182	0.5	TTC	GCC	T	-	G	G
	25255	0.5	TTC	GCC	T	-	G	G
	24228	0.5	TTC	GAC	T	-	G	T
C	18853	1	TTC	AAC	T	-	A	T
	19081	1	TTC	AAC	T	-	A	T
	19316	1	TTC	GAC	T	-	G	T
	19132	2	TTC	GGC	T	-	G	C
	19677	2	TTC	AAC	T	-	A	T
	19986	2	TTC	AAC	T	-	A	T
	20419	2	TTC	GGC	T	-	G	C
	18475	4	TTC	GCC	T	-	G	G
	19145	4	TTC	GCC	T	-	G	G
	21146	4	TTC	GGC	T	-	G	C
	19289	8	TTC	GGC	T	-	G	C
	19330	8	TTC	GGC	T	-	G	C
	19333	8	TTC	GGC	T	-	G	C
	21145	8	TTC	GCC	T	-	G	G
	18610	16	TTC	GGC	T	-	G	C
	18907	16	TTC	AAC	T	-	A	T
	19703	16	TTC	GGC	T	-	G	C
	19707	16	TTC	GGC	T	-	G	C
	19709	16	TTC	GGC	T	-	G	C
	19328	32	TTC	GGC	T	-	G	C

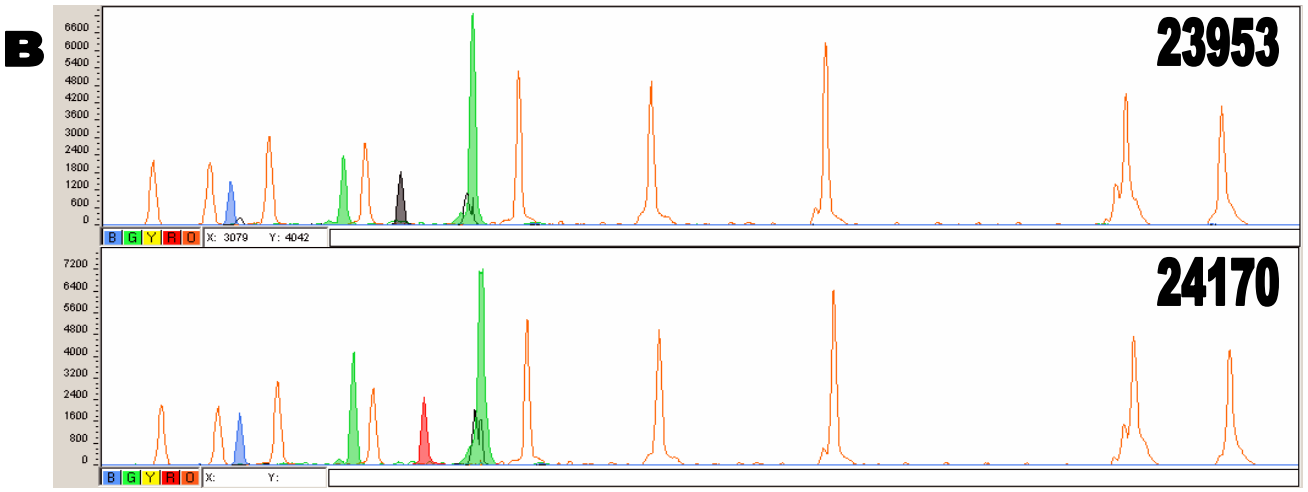
Multiplex electropherograms are shown in Figure 17 for the eight *parC* oligonucleotides for six strains: two ciprofloxacin susceptible, two intermediate resistant, and two resistant. For FA1090, six peaks were observed representing SNPs identified by oligonucleotides 86CF, 91CR2, 91CF1, 88CR, 87CR1, and 87CF1 respectively. For all other strains four peaks were observed representing SNPs identified by oligonucleotides 86CF, 88CR, either 87CR1 or 87CR2 and either 87CF1 or 87CF2 respectively. These strains are represented because they depict all possible SNP combinations observed for *parC*. FA1090 in the top panel is a wild type strain with no SNPs, while the remaining strains possess at least one the SNPs listed in Table 9. Wild type FA1090 shows a blue peak at "1" in Figure 17, Block A indicating 86CF identifying a G nucleotide was added to the end of this oligonucleotide. Additionally, the red peak at "2" in Figure 17, Block A indicates 91CR2 identifying a T nucleotide was added to the 3' end of this oligonucleotide while a blue peak at "3" indicates 91CF1 identifying a G nucleotide, a green peak at "4" indicates 88CR identifying an A nucleotide, a black peak at "5" indicates 87CR1 identifying a C nucleotide, and a green peak at "6" indicates 87CF1 identifying an A nucleotide. The *parC* QRDRs of all 60 strains were sequenced and amino acid codons 86, 87, 88 and 91 are displayed in Table 11 verifying the results obtained by capillary electrophoresis. From Table 9, the most common *parC* SNPs that were found In this study were D86N (GAC→AAC) in 7 strains (11.7%), S87R (AGT→CGT) in 7 strains (11.7%), and S87N (AGT→ATT) in 6 strains (10%). Only 1 strain had the S88P (TCC→CCC) mutation and none contained the S87I (AGT→ATT), S88R (TCC→CGC), E91K (GAG→AAG), E91G (GAG→GGG) and

E91Q (GAG→CAG) mutations. Also, as the ciprofloxacin MICs increased, so did the numbers of SNPs in both *gyrA* and *parC* (Table 10).

Finally, a multiplex electropherogram is shown in Figure 18 for the two *mtr* oligonucleotides for strain 19081 showing that a multiplex combination of the MA and MT oligonucleotides is possible. In 19081, MA detected a G nucleotide and MT identified a C nucleotide indicating that it contains the single deletion but not the dinucleotide insertion. Multiplexing was not performed with the *mtr* oligonucleotides on all 60 *N. gonorrhoeae* strains.

Figure 17: Electropherograms for multiplex SNPs of *parC* for multiple strains. Standard peaks are coloured in orange with their sizes in bp labeled. Representative strains from the sensitive, intermediate resistant, and resistant groups show all observable SNP combinations as illustrated in block A, block B and block C respectively. Peaks at 1, 2, 3, 4, 5, and 6 correspond to oligonucleotides 86CF, 91CR2, 91CF1, 88CR, 87CR1 and 87CF1 respectively detecting SNPs for strain FA1090 whereas each coloured peak corresponds to a specific nucleotide: Black-C, Blue-G, Green-A, and Red-T. Oligonucleotides 86CF, 88CR, 87CR1 or 87CR2, and 87CF1 or 87CF2 respectively detected SNPs for all other strains.


A
FA1090
15
20
25
35
6
50
62
80
110
120
1
2
3
4
5
24939


B
23953
24170

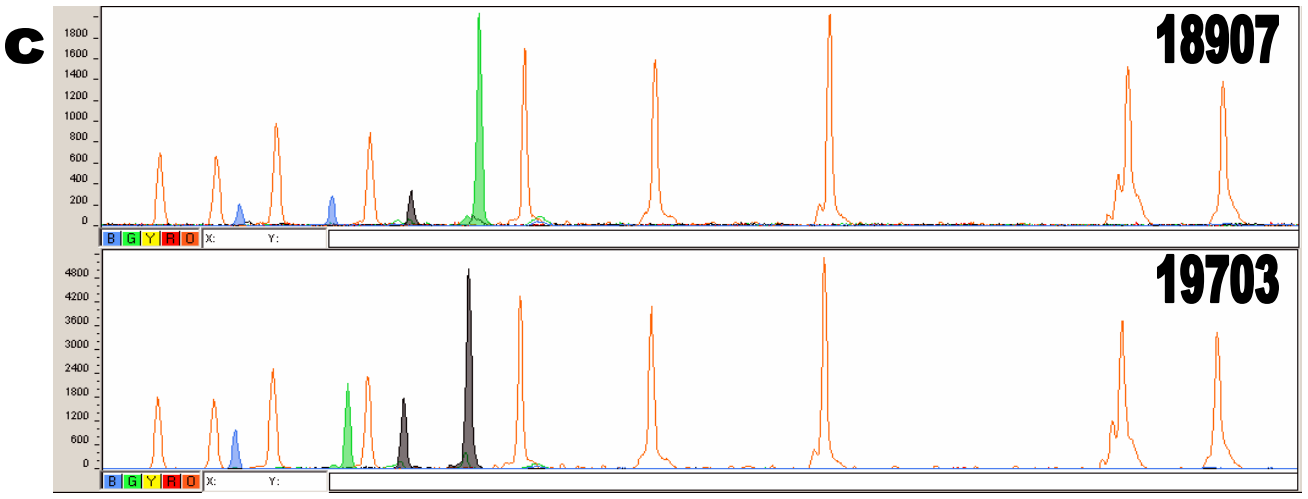
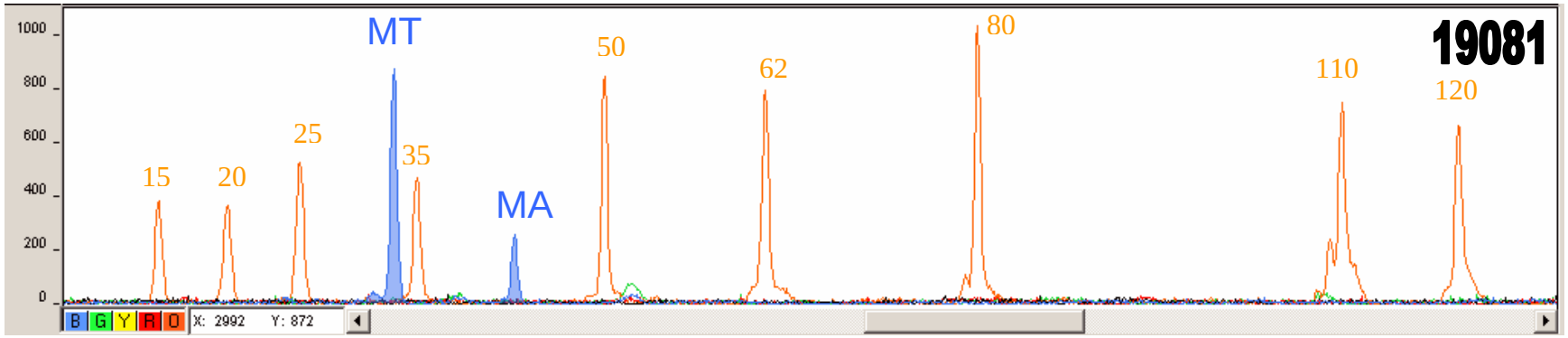

C
18907
19703

Table 11: *N. gonorrhoeae* strains used including their ciprofloxacin MICs (μg/mL), *parC* mutations at codons 86, 87, 88, and 91 and the respective SNPs observed with capillary electrophoresis.

Footnote: Block A represents ciprofloxacin susceptible strains (MIC<0.25 μg/mL), Block B represents ciprofloxacin intermediate resistant strains (MIC 0.25-0.5 μg/mL) and Block C represents ciprofloxacin resistant strains (MIC>1 μg/mL).
[a] SNPs at codons 86, 87, 88 and 91 are highlighted in yellow.
[b] 86CF identified the SNP at the first position of codon 86.
[c] only one of oligonucleotides 87CF1 and 87CF2 was able to identify the SNP at the first position of codon 87.
[d] only one of oligonucleotides 87CR1 and 87CR2 was able to identify the SNP at the second position of codon 87 (reverse strand).
[e] 88CR identified the SNP at the first position of codon 88 (reverse strand).
[f] 91CF1 identified the SNP at the first position of codon 91.
[g] 91CR2 identified the SNP at the second position of codon 91 (reverse strand).

	Strain	MIC	86[a]	87[a]	88[a]	91[a]	86CF[b]	87CF1[c]	87CF2[c]	87CR1[d]	87CR2[d]	88CR[e]	91CF1[f]	91CR2[g]
	FA1090	0.004	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
	25714	0.004	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
	25727	0.004	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
	25713	0.008	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
	25716	0.008	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
	25718	0.008	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
	25719	0.008	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
	25729	0.016	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
	25733	0.016	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
	25742	0.016	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
	24967	0.032	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
A	24974	0.032	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
	25722	0.032	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
	23895	0.064	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
	24795	0.064	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
	24939	0.064	GAC	CGT	TCC	GAG	G	C	-	C	-	A	G	T
	25450	0.125	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
	25717	0.125	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
	25736	0.125	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
	25737	0.125	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
	23937	0.25	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
	23953	0.25	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
	24058	0.25	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
	24059	0.25	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
	24118	0.25	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
	24146	0.25	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
	24170	0.25	GAC	AAT	TCC	GAG	G	A	-	T	-	A	G	T
	24178	0.25	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
	24179	0.25	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
	24713	0.25	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
	23536	0.5	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
B	24078	0.5	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
	24228	0.5	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
	24364	0.5	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
	24380	0.5	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
	24437	0.5	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
	24489	0.5	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
	25092	0.5	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
	25182	0.5	GAC	AAT	TCC	GAG	G	A	-	T	-	A	G	T
	25255	0.5	GAC	AAT	TCC	GAG	G	A	-	T	-	A	G	T
	18853	1	AAC	AGT	TCC	GAG	A	-	A	C	-	A	G	T
	19081	1	AAC	AGT	TCC	GAG	A	-	A	C	-	A	G	T
	19316	1	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
	19132	2	GAC	AAT	TCC	GAG	G	-	A	T	-	A	G	T
	19677	2	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
	19986	2	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
	20419	2	AAC	AGT	TCC	GAG	A	-	A	C	-	A	G	T
	18475	4	GAC	AAT	TCC	GAG	G	A	-	T	-	A	G	T
	19145	4	AAC	AGT	TCC	GAG	A	-	A	C	-	A	G	T
	21146	4	GAC	CGT	TCC	GAG	G	C	-	C	-	A	G	T
	19289	8	GAC	CGT	TCC	GAG	G	C	-	C	-	A	G	T
	19330	8	AAC	AGT	TCC	GAG	A	-	A	C	-	A	G	T
C	19333	8	GAC	AGT	TCC	GAG	G	A	-	C	-	A	G	T
	21145	8	GAC	AAT	TCC	GAG	G	A	-	T	-	A	G	T
	18610	16	AAC	AGT	TCC	GAG	A	-	A	C	-	A	G	T
	18907	16	GAC	AGT	CCC	GAG	A	-	A	-	C	G	G	T
	19703	16	GAC	CGT	TCC	GAG	G	C	-	C	-	A	G	T
	19707	16	GAC	CGT	TCC	GAG	G	C	-	C	-	A	G	T
	19709	16	GAC	CGT	TCC	GAG	G	C	-	C	-	A	G	T
	19328	32	GAC	CGT	TCC	GAG	G	C	-	C	-	A	G	T

Figure 18: Electropherogram for multiplex SNPs of *mtr* for strain 19081. Standard peaks are coloured in orange with their sizes in bp labeled. The *mtr* oligonucleotides MT at position 33 bp and MA at position 40 bp both detected G nucleotides (blue peaks).


19081
MT
MA
15
20
25
35
50
62
80
110
120
1000
800
600
400
200
0
B G Y R O
X: 2992 Y: 872

3.2.3 Detection of *N. gonorrhoeae* SNPs in clinical samples

The next step in SNP detection, knowing that it worked effectively in laboratory isolates, was to determine if it could be applied to detect SNPs in clinical isolates. Therefore, five urine samples were obtained from each of three male individuals during a 24 hr period to take into consideration the variability of urine concentrations, pH's, inhibitors, hormones, and other proteins (114). Aliquots of each urine sample were pooled and infected with a loopful of an overnight culture of *N. gonorrhoeae* strain 19703 to ensure mostly T1 and T2 piliated cells (98). Urine cultures were incubated and DNA was isolated and processed as described in 2.12 DNA Isolation in Urine Processing at 1, 4 and 24 hr intervals during growth to guarantee active infection of gonococci in urine epithelial cells (49). Active infection is important because this imitates how *N. gonorrhoeae* infects its host *in vivo* to propagate and how *N. gonorrhoeae* would present itself clinically within epithelial cells (11).

Figure 19 shows a PCR using *gyrA* and *parC* primers (Table 4) to determine if *N. gonorrhoeae* DNA could be extracted from urine and visualized at 1 hr and 4 hr intervals after inoculation. Data for 24 hr is not shown. PCR products for *gyrA*, 266 bp and *parC*, 331 bp were detected from the urine samples at both time intervals, with the *parC* PCR products at 4 hr producing a much brighter band than other samples. The PCR products from these experiments were then processed for capillary electrophoresis as multiplex reactions using the oligonucleotides from Table 5 as described in Sections 2.8 Multiplex SNaPshot Reactions and 2.10 Capillary Gel

Electrophoresis. Figure 20 shows that SNPs were detectable from urine samples containing *N. gonorrhoeae* at both 1hr and 4 hrs. Detection with the *gyrA* oligonucleotides was much better at both time points than the detection of *parC* oligonucleotides most likely due to less competition for DNA binding by multiplexing with fewer oligonucleotides. Also, detection with both the *gyrA* and *parC* oligonucleotides lead to more background noise than was observed for multiplex reactions with laboratory isolates. However, this does not appear to be due to urine components (inhibitors, hormones, and other proteins) as this background is present within the GCMB grown control cells as well.

Urine was also infected with various other bacterial species that may be present in natural urine samples and processed exactly as described for *N. gonorrhoeae*, to ensure this technique was specific for gonococcal infections in mixed bacterial infections. Therefore, strains of *S. aureus, L. lactis, L. acidophilus*, and *E. coli* as well as closely related species *M. osloensis* were examined. DNA isolated from each species was amplified with *gyrA* and *parC* specific primers (Table 4), run on an agarose gel and set up for multiplex capillary electrophoresis with *gyrA* and *parC* oligonucleotides (Table 5). PCR products did not appear on the agarose gel for either 1 hr, 4 hr, or 24 hr cultures, and similarly no peaks were detected during capillary electrophoresis for any time point indicating a specificity for *N. gonorrhoeae* (data not shown). However, one of the limitations was that the nature of fluoroquniolone resistance in these species was unknown. Further analysis of strains carrying similar antibiotic resistance to *N. gonorrhoeae* would be needed to unequivocally state that this technique was *N. gonorrhoeae* specific.

Figure 19: The polymerase chain reaction for the detection of genes, *gyrA* and *parC*, in *N. gonorrhoeae* strain 19703 isolated from urine. *gyrA* PCR product is 266 bp and *parC* PCR product is 331 bp. A 100 bp ladder (Invitrogen) in lane 1 was used as a sizing standard. Lanes 2, 4, 6 and 8 contain DNA controls isolated from GCMB while lanes 3, 5, 7 and 9 contain DNA isolated from urine. Urine cultures inoculated with *N. gonorrhoeae* for 1hr (lanes 2, 3, 6, 7) and 4hr (4, 5, 8, 9) before DNA extraction.

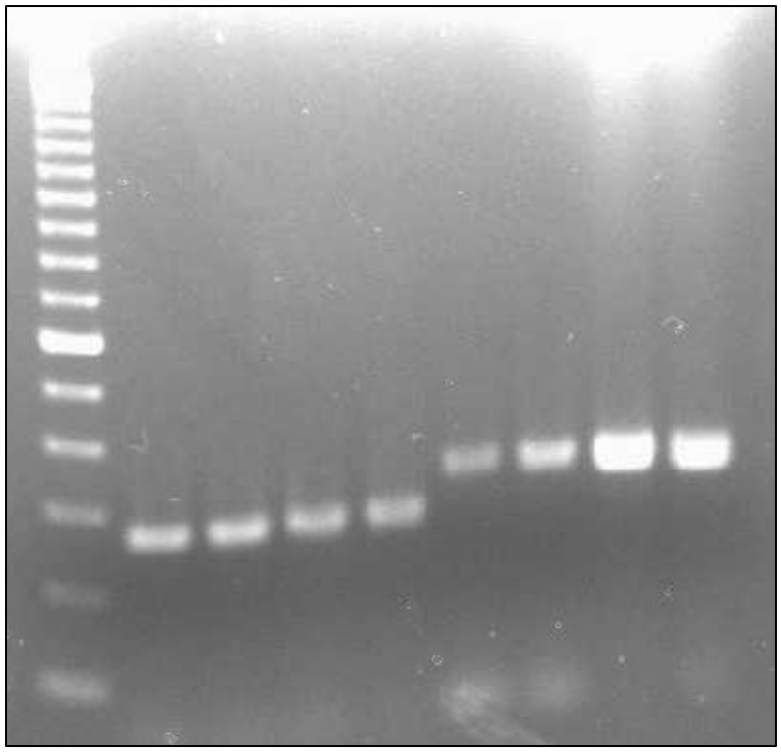
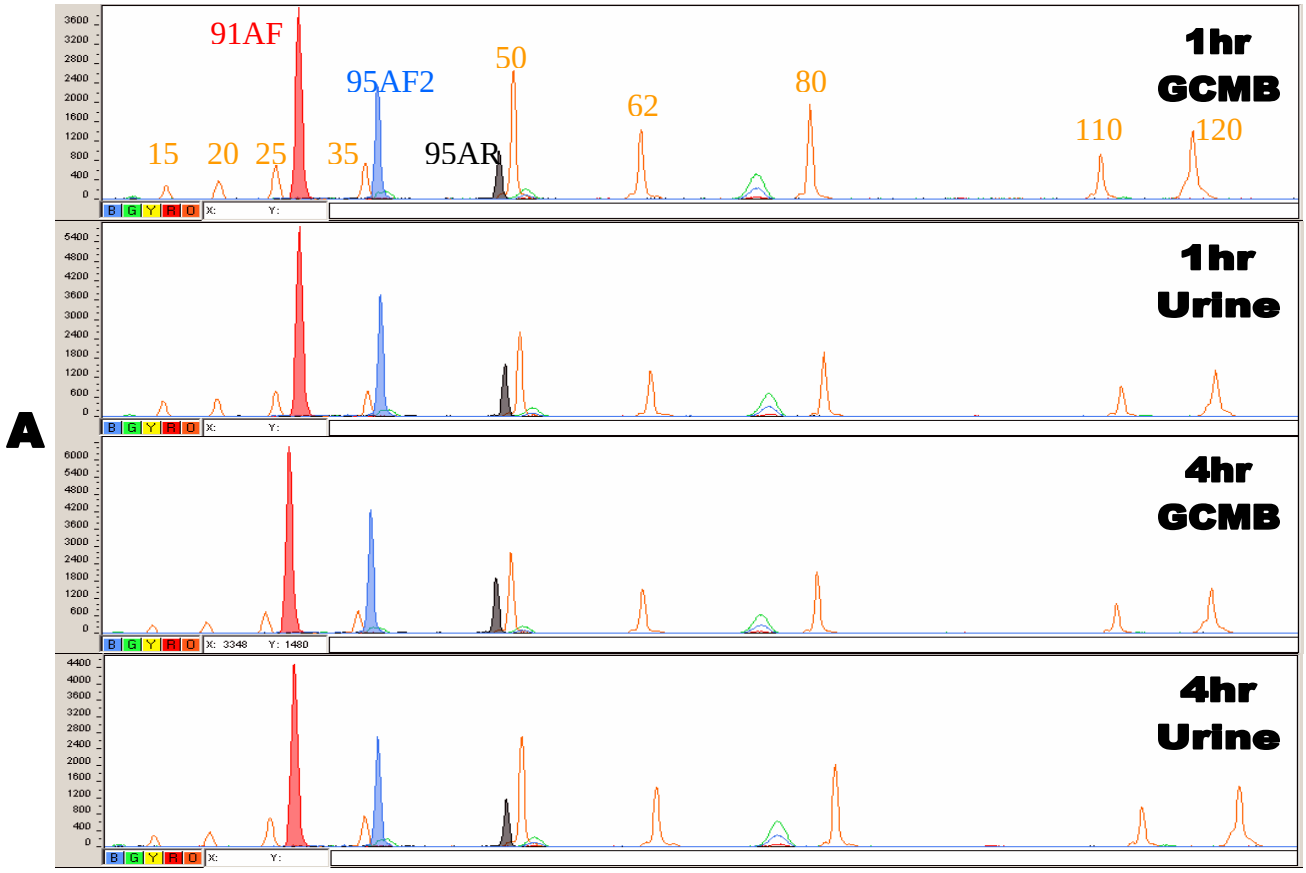
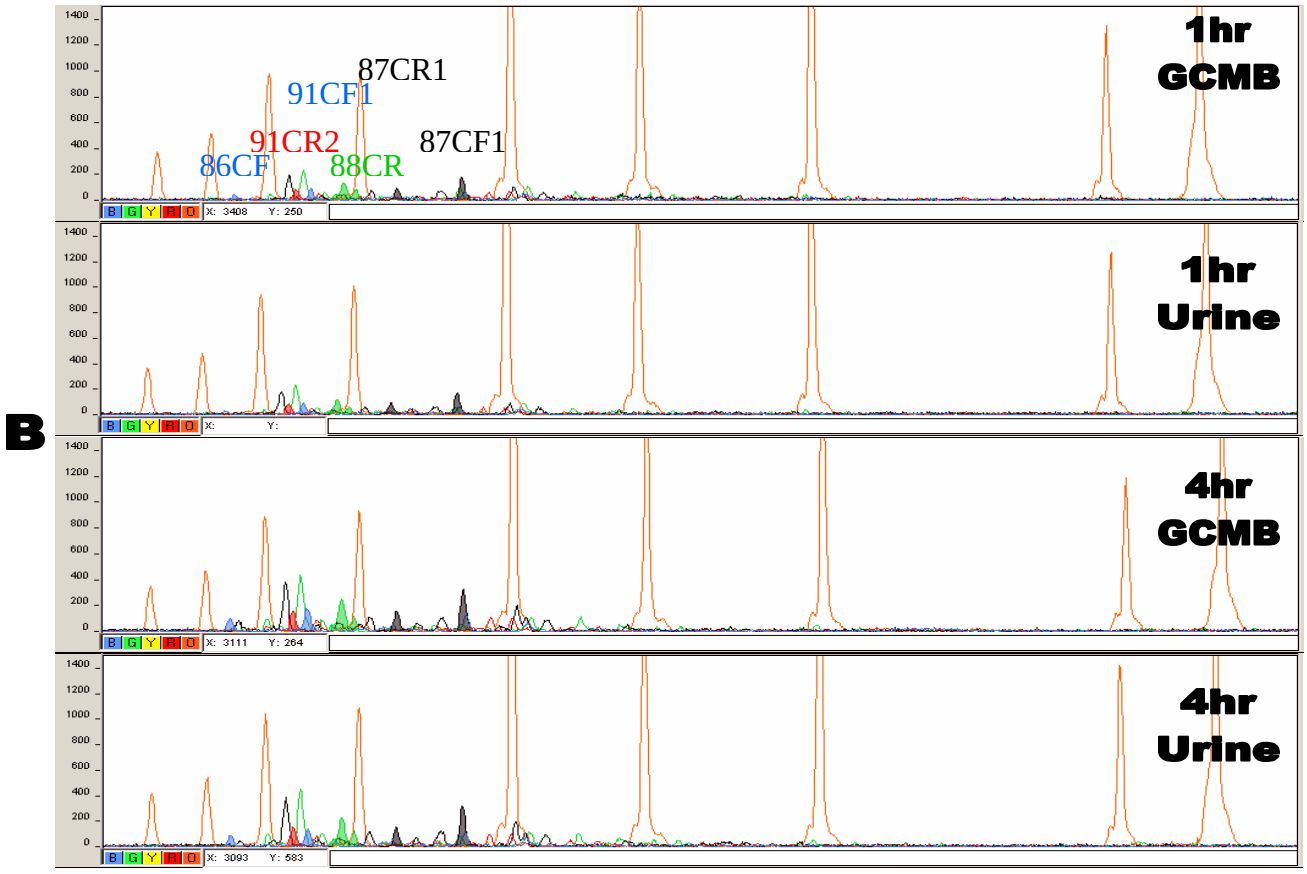

331bp
266bp
1 2 3 4 5 6 7 8 9

Figure 20: Electropherograms for multiplex SNPs of *gyrA* and *parC* for *N. gonorrhoeae* strain 19703 isolated from urine. Standard peaks are coloured in orange with their sizes in bp labeled. Panel A depicts *gyrA* oligonucleotides from left to right: 91AF, 95AF2 and 95AR under the conditions described for each electropherogram. Panel B depicts *parC* oligonucleotides from highlighted peaks left to right: 86CF, 91CR2, 91CF1, 88CR, 87CR1, and 87CF1 under the conditions described for each electropherogram. 1 hr and 4 hr indicates how long the urine cultures were inoculated with *N. gonorrhoeae* before DNA extraction. GCMB (control) and Urine indicate the media conditions that cultures were grown in.


A
91AF
95AF2
95AR
15
20
25
35
50
62
80
110
120
1hr GCMB
1hr Urine
4hr GCMB
4hr Urine


B
87CR1
91CF1
91CR2
87CF1
86CF
88CR
1hr GCMB
1hr Urine
4hr GCMB
4hr Urine

3.2.4 Sensitivity of SNP detection in *N. gonorrhoeae* for clinical samples

It has been shown that SNP detection can be applied to clinical isolates of *N. gonorrhoeae*. However, the question of SNP detection sensitivity has not been addressed. Therefore, urine cultures were set up as previously described in 3.2.3 Detection of *N. gonorrhoeae* SNPs in Clinical Samples with the modification that instead of adding a loopful of overnight *N. gonorrhoeae* cells to urine cultures, concentrations of *N. gonorrhoeae* cells ranging from 10^2 cells/mL to 10^8 cells/mL were added to urine cultures for the 1, 4 and 24 hr timed intervals. DNA from these samples was obtained by a crude boil extraction.

Figure 21 shows a PCR using *gyrA* and *parC* primers (Table 4) to determine which cell concentrations produce an observable PCR product at 4 hr and 24 hr post-infection. Data for 1 hr is not shown. In *gyrA*, amplified product was seen only at a cell concentration of 10^8 cells/mL in both 4 hr and 24 hr cultures. In *parC*, amplified product was observed in a cell concentration of 10^8 cells/mL at 4 hr and faintly observed at cell concentrations of 10^7 cells/mL and 10^8 cells/mL in a 24 hr culture. All of the PCR products from these experiments were processed for capillary electrophoresis using a single *gyrA* oligonucleotide 95AF2 and a single *parC* oligonucleotide 87CR1 to simply detect at what cell concentrations SNP detection could occur. Figure 22 depicts only the electropherograms that could detect a peak. Panel A shows that SNP detection in *gyrA* could occur at a cell concentration of 10^8 cells/mL at both 4 hr and 24 hr post-infection and at no other cell concentration or time point (data not shown). This corresponds to the results

seen for *gyrA* detection by agarose gel (Figure 21). Panel B shows that SNP detection in *parC* could occur at cell concentration of 10^8 cells/mL in 4 hr cultures and in cell concentrations of 10^7 cells/mL and 10^8 cells/mL in 24 hr cultures, but not within any other cell concentration or time point (data not shown). This also corresponds to the results seen for *parC* detection by agarose gel (Figure 22). Comparing Figure 21, Panel B for 24 hr urine cultures, it can be observed that more amplicon is present in the 10^7 cells/mL (lane 8) than in the 10^8 cells/mL (lane 9). This result is analogous to capillary electrophoresis results shown in Figure 22, Panel B where the peaks in 24 hr culture samples for 10^7 cells/mL and 10^8 cells/mL show the same trend; the fluorescence signal in the 10^7 cells/mL culture is more pronounced than in a culture of 10^8 cells/mL. This difference may be attributed to the cell density of the 10^8 cells/mL culture having more DNA than that of the 10^7 cells/mL culture. Therefore, a PCR would be oversaturated with DNA from these samples and less amplicon would be made. In addition, it appears amplicon from Figure 21, Panel B for the 4hr 10^8 cells/mL (lane 9) is more abundant than the amplicon from either 24 hr 10^7 cells/mL or 10^8 cells/mL cultures (lanes 8, 9). Differences between fluorescent signals in Figure 22, Panel B are also seen with these cultures. This may be a result of isolating cells during the exponential phase of cell growth. In this case, an optimal ratio of DNA to PCR components may have been reached. Ultimately, SNP detection appears to be sensitive up to a *N. gonorrhoeae* concentration of 10^8 cells/mL after a 4 hr incubation in urine. If incubation proceeds to 24 hr, detection may be possible with 10^7 cells/mL. Running

an agarose gel of amplified DNA from urine samples is also indicative of SNP detection using capillary electrophoresis.

Figure 21: The lower limits of detection for genes, *gyrA* and *parC* in *N. gonorrhoeae* strain 19703 isolated from urine as detected using polymerase chain reaction. *gyrA* PCR product is 266 bp and *parC* PCR product is 331 bp. Panel A shows the detection limits for *gyrA* DNA at 4hr and 24hr while panel B shows the detection limits for *parC*. A 100bp ladder (Invitrogen) in lane 1 was used as a sizing standard. Lane 2 represents 19703 DNA isolated from GCMB agar at the respective time as a positive control. Lanes 3 to 9 represent the ten-fold concentration of *N. gonorrhoeae* cells incubated in urine ranging from 10^2 cells/mL to 10^8 cells/mL.

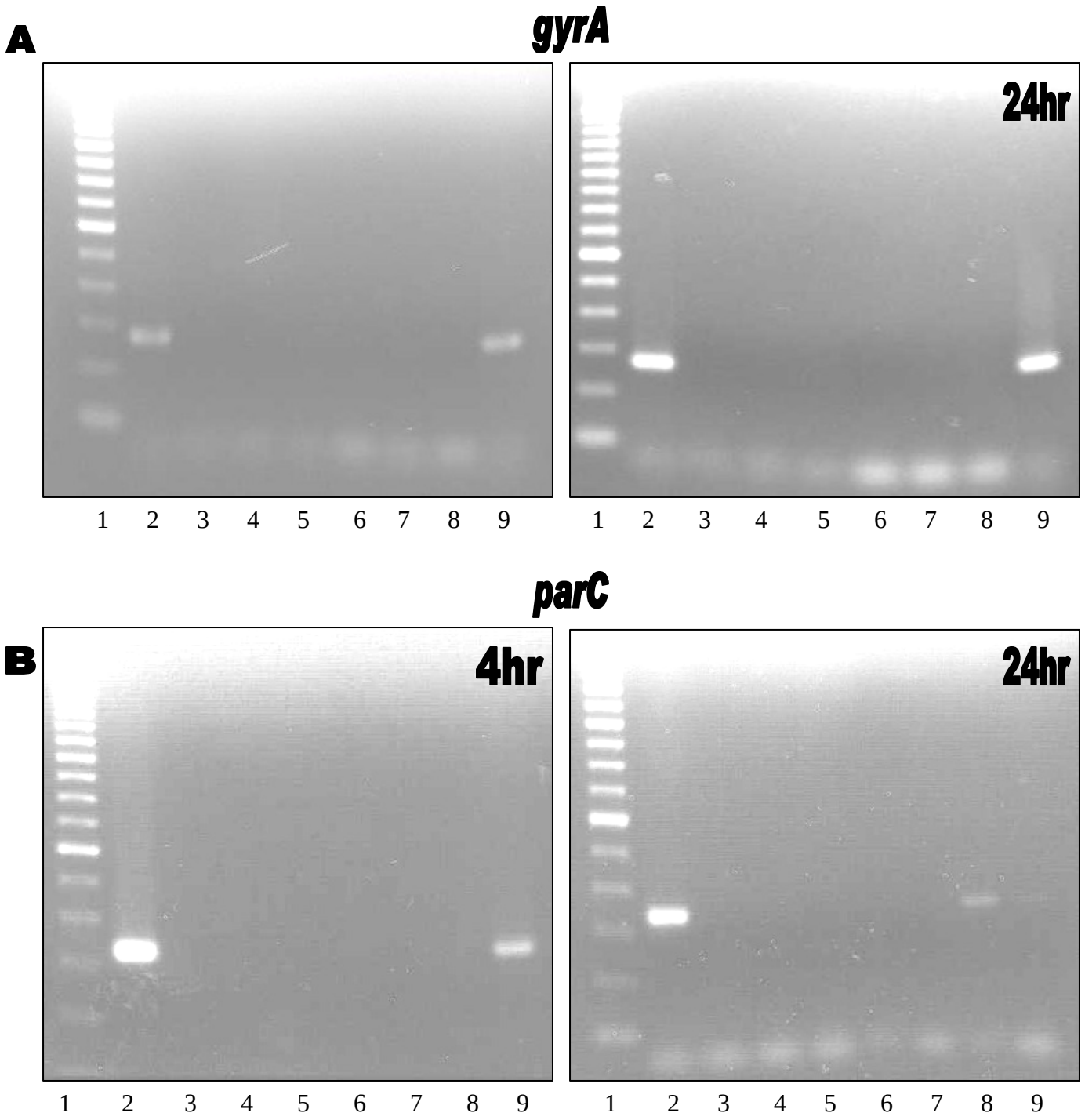
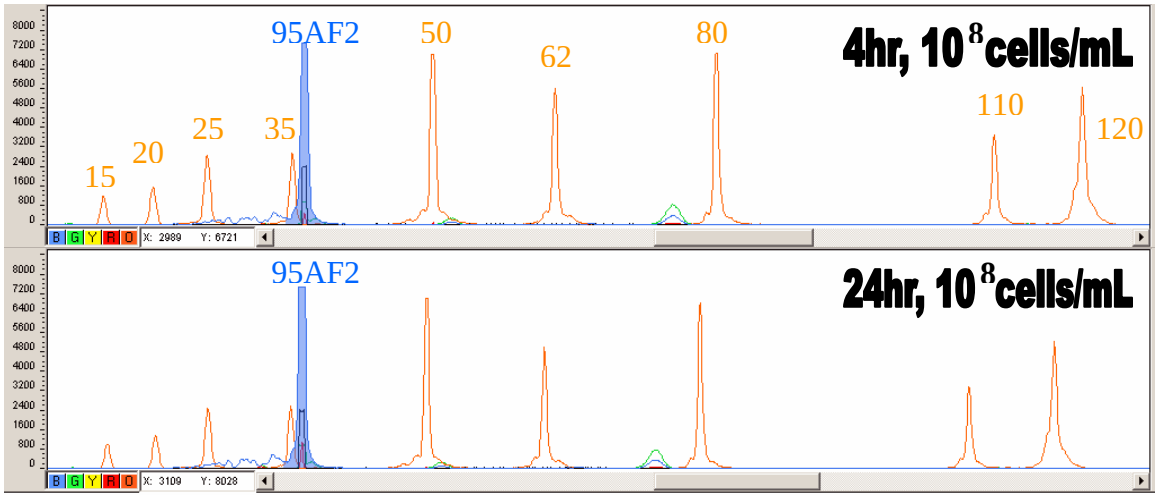
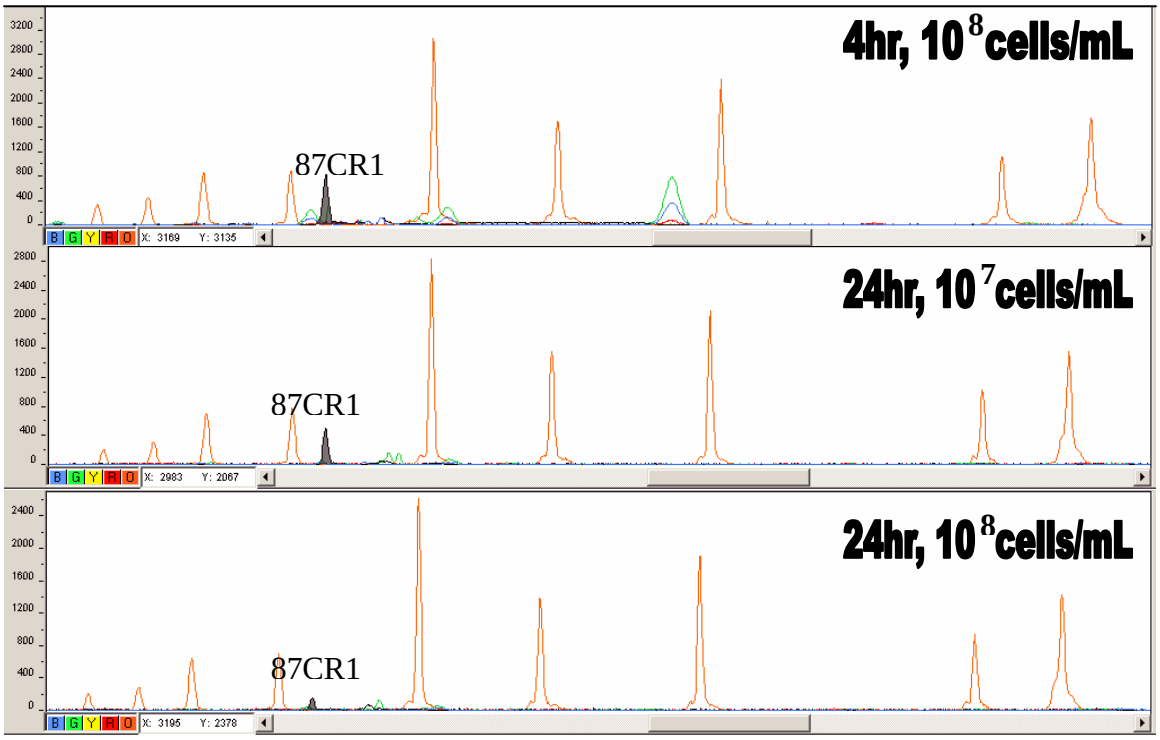

A
gyrA
24hr
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9
B
parC
4hr
24hr
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9

Figure 22: The lower limits of detection for genes *gyrA* and *parC* in *N. gonorrhoeae* strain 19703 isolated from urine as detected by capillary electrophoresis. Standard peaks are coloured in orange with their sizes in bp labeled. Panel A depicts oligonucleotide 95AF2 detected in *gyrA* at various time points and cell concentrations of *N. gonorrhoeae*. Panel B depicts oligonucleotide 87CR1 detected in *parC* at various timepoints and cell concentrations of *N. gonorrhoeae*.

A


95AF2
50
62
80
4hr, 10 8 cells/mL
110
120
25
35
20
15
95AF2
24hr, 10 8 cells/mL

B


4hr, 10 8 cells/mL
87CR1
24hr, 10 7 cells/mL
87CR1
24hr, 10 8 cells/mL
87CR1

4. DISCUSSION

Treating gonorrhea has been challenged by the rapid emergence of gonococcal strains with altered antimicrobial susceptibilities (33). With the ability to treat gonorrhea with sulfonamides, penicillins, tetracyclines, and erythromycin being severely hindered in the late 1980's, Health Canada and the CDC recommended fluoroquinolones or cephalosporin use to treat uncomplicated gonococcal infections (33, 62, 75). In the past decade, resistance to the fluoroquinolones has risen worldwide (Table 2), whereas plasmid-mediated penicillin resistance and chromosomally mediated tetracycline resistance has declined significantly (104). Nevertheless, fluoroquinolone resistance is currently spreading and is more frequently seen in PPNG strains or strains with chromosomal resistance, meaning these strains are becoming multi-resistant (7). In fact, among the antimicrobials available for treatment, the third generation cephalosporins are the only agents to which *N. gonorrhoeae* is not resistant to (7).

Most of the mechanisms of resistance are well characterized therefore, it is appropriate to use *N. gonorrhoeae* as a model to develop laboratory techniques to detect some of these bacterial resistant mechanisms. Chromosome-mediated antimicrobial resistance in *Neisseria gonorrhoeae* can occur as a direct result of three main processes including alteration of target enzymes, changes in transmembrane transport and active extrusion via efflux function. Single nucleotide polymorphisms within target enzymes DNA gyrase (*gyrA*), topoisomerase IV (*parC*), as well as mutations in the promoter regions of the *mtr* and *norM* efflux pumps are mostly responsible for the increased resistance in antimicrobials in *N. gonorrhoeae*

(72, 94, 126, 127). In particular, mutations solely in the *gyrA* subunit, confer low level fluoroquinolone resistance (MIC 0.25-0.5 μg/mL) while mutations within both *gyrA* and *parC* subunits result in higher level fluoroquinolone resistance (MIC ≥ 1 μg/mL) (7). Combination of mutations in *gyrA* and *parC* are required for resistance causing treatment failure. Additionally mutations in the *mtrR* and *norM* promoters result in increased expression of the *mtrCDE* and *norM* genes, leading to elevated levels of multidrug resistance to penicillin, the macrolides azithromycin and erythromycin, and the fluoroquinolones, ciprofloxacin and norfloxacin (94, 97, 127). Mutations can often occur in the *gyrB* subunit of DNA gyrase, but mutations in this gene are characteristic to laboratory strains and lack clinical correlation to treatment failure (7). In this study, we looked at explaining the differences between the ciprofloxacin MICs of two strains that we thought may be due to SNPs in *norM*, as well as analyzing known SNPs using the SNaPshot method to rapidly predict resistant isolates.

NorM function in ciprofloxacin resistant strains. Efflux pumps primarily confer low-level protection that facilitates the initial survival of the organism in order to provide it with the opportunity to acquire high-level resistance from another mechanism (45). Thus we thought that the 4-fold difference in ciprofloxacin MIC between strains 19703 (MIC 16 μg/mL) and 21146 (MIC 4 μg/mL) may be due to a fluoroquinolone pump in addition to the high-level resistance acquiring mutations in *gyrA* and *parC* that both strains shared. Sequencing analysis of the *norM* gene and promoter revealed a novel T deletion within the *norM* promoter. The only other

published literature on the *N. gonorrhoeae* NorM pump by Rouquette-Laughlin *et al*. identified two mutations within the promoter, one in a putative –35 bp promoter region and another within a likely RBS which were not seen in our strains (94). Intracellular drug accumulation studies using the protonophore CCCP, showed no ciprofloxacin MIC differences in cultures subjected to CCCP exposure and not subjected to CCCP indicating that the proton motive force was not responsible for causing the 4-fold difference between strains. In addition, transformational studies using two distinct transformation protocols employed 19703 and 21146 DNA into a wild type strain, gave ten constructs of elevated ciprofloxacin MICs (2 to 16 fold). However, when these constructs were sequenced, not one accrued the expected T deletion that would give cause for the increase in MIC. No other mutations were found when the *gyrA* and *parC* QRDRs of these constructs were sequenced or within a 2286 bp region of the *gyrA* gene. It can be concluded by our data, that the NorM efflux pump was not the source of the 2-fold increase in ciprofloxacin resistance. Instead, this increase may be a result of several factors. First, the explanation may simply lie in the definition of MIC. A MIC is the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in an agar or broth dilution susceptibility test (84). However, the MIC does not represent an absolute value. The 'true' MIC is somewhere between the lowest test concentration that inhibits growth to the next lowest test concentration and generally, the accepted reproducibility of MICs is within one twofold dilution of the actual end point (84). Therefore, strain 19703 with a MIC of 4 µg/mL may by definition actually have a MIC of 2 µg/mL or 8 µg/mL and strain 21146 with a MIC of

16 µg/mL may actually have a MIC of 8 µg/mL or 32 µg/mL. Since this gives both strains the possibility of having a MIC of 8 µg/mL then perhaps the differences between these two strains is non-existent by the definition of MIC. Putting this justification aside, there may be molecular reasons for the noticeable difference. There may be an unidentified ABC efflux pump in *N. gonorrhoeae* capable of extruding fluoroquinolones, which would explain why CCCP had no effect on MIC values. The most likely explanation though might be a result of the disruption of the outer membrane to enhance susceptibility to antimicrobials. *N. gonorrhoeae's* LOS produces an anionic charge and a cross-linked bridge of the core region consisting of the tightly packed fatty acyl chains of lipid A that can provide a barrier to hydrophobic agents (92). Mutations to the LOS can allow these agents to enter the cell. Although, entry of hydrophilic antimicrobials such as fluoroquinolones occurs through channels formed by porin proteins (1). Therefore, the number of porin channels in strains 19703 and 21146 could be the reason the ciprofloxacin susceptibilities vary (92). Another possibility could be due to *N. gonorrhoeae's* Opa (opacity-associated) proteins. Opa proteins are one of several outer membrane components that undergo frequent antigenic and phase variation (28). An individual gonococcus possesses about a dozen *opa* genes and can express anywhere from 0 to 3 or more of them at one time that can help prevent antimicrobials from entering the cell based on their conformation (29). Thus, any one or a combination of the above outer membrane components can explain the MIC differences between 19703 and 21146. This could be confirmed by differential expression of the porin and Opa

proteins from *N. gonorrhoeae* by Western blot analysis to determine if any of these proteins are present and active in strains 19703 and 21146.

SNP detection using SNaPshot. We have described a SNP detection protocol that utilizes oligonucleotide primers to detect point mutations in *N. gonorrhoeae* to antimicrobial resistance targets *gyrA, parC, mtr* and *norM*. However, this protocol could easily be adapted to detect more targets at a higher throughput. The number of oligonucleotide primers can be expanded as new SNPs are identified. With many diagnostic and public health laboratories moving towards molecular methods for diagnosing disease, fewer cultures are available for susceptibility testing. The SNaPshot technique used to identify point mutations in resistance genes and other genetic targets, could be complimentary to these methods. To our knowledge this is the first time that the SNaPshot method has been used to detect fluoroquinolone resistance in *N. gonorrhoeae* specimens or antimicrobial resistance in any bacterial organism for that matter. The successful implementation of this system depends heavily on oligonucleotide design. Briefly, it is imperative that the 3' end of the oligonucleotide ends adjacent to the SNP to be detected. For any oligonucleotides that overlap neighbouring SNPs, we adopted a system whereby wild type nucleotides were incorporated at all SNP locations unless the overlapped SNP was within 5 bp of the oligonucleotide's 3' end. Using these arbitrary designations it was possible to detect SNPs in *gyrA* at codons 91 and 95, in *parC* at codons 86, 87, 88, and 91 and within the *mtr* promoter region. *norM* SNPs were not identifiable using this method. Booth *et al*. noticed that when designing microarray oligonucleotides

(18 to 24 bp) for deer mouse mitochondrial DNA, AT rich regions containing the polymorphism of interest (GC content 20-33%) resulted in poor hybridization with virtually undetectable signal (15). The *norM* oligonucleotides designed were between 16 to 50 bp with GC contents ranging from 33-61% and when tested with SNaPshot and capillary electrophoresis produced multiple peaks at varying lengths (results not shown). Compared to the GC content of *gyrA* (46-60%), *parC* (57-70%) and *mtr* (33-43%), the *norM* oligonucleotides GC content was well within these ranges. However, looking at the regions containing the polymorphism, the *norM* sequence contained many long AT rich regions in the form of runs of A's, runs of T's and runs of AT consecutive sequences compared to the *gyrA, parC* and *mtr* regions. The incomplete hybridization of these oligonucleotides to their respective regions, that would result from AT rich sequences, is consistent with the pattern of multiple peaks observed with capillary electrophoresis. Therefore, one of the limitations of the SNaPshot technique is that oligonucleotides cannot be designed over highly rich regions of AT nucleotides in the form of consecutive strings.

Identification of single and multiplex SNPs were both possible using the SNaPshot technique. SNPs observed with the four *gyrA* oligonucleotides include S91F, S91Y, D95N, D95G and D95A, while the eight *parC* oligonucleotides could detect D86N, S87N, S87R, and S88P mutations. Sequencing analysis of 60 ciprofloxacin susceptible, intermediate resistant, and resistant *N. gonorrhoeae* strains confirmed that no strains contained the *gyrA* mutation S91C and *parC* mutations S87I, S88R. E91K, E91G, and E91Q. Thus the *gyrA* and *parC* oligonucleotides did not detect these SNPs although they are designed to do so if

ever encountered. Among the 60 strains tested, 30% (6/20) of the ciprofloxacin susceptible strains (MIC < 0. 25 µg/mL) had at least a single *gyrA* SNP detected and 100% of the intermediate resistant (MIC 0.25-0.5 µg/mL) (20/20) and resistant (MIC ≥ 1 µg/mL) (20/20) strains had a least a single *gyrA* mutation with 15% (3/20) of the intermediate resistant strains and 95% (19/20) of the resistant strains having two *gyrA* mutations. Interestingly, of strains that identified two *gyrA* mutations in the intermediate resistance category, 33% (2/6) contained at least one *parC* SNP and 84% (16/19) of the resistant strains also contained at least one *parC* SNP. Additional SNPs in *gyrA* A67S, A75S, A84P, V120L and G85C, A92G, F100Y, R116H and R116L in *parC* which are less common, identified in countries such as Japan, India, Denmark, and the United States were not taken into consideration for this study since sequencing analysis verified the absence of these SNPs in all 60 Canadian strains (23, 100, 103, 104, 109). However, in future studies, oligonucleotides can be designed to identify these SNPs. Oligonucleotides were also used to detect a single A deletion and dinucleotide TT insertion in the *mtrR* promoter with success. Furthermore, SNP detection was observed in clinical samples of spiked urine. Various *N. gonorrhoeae* cell concentrations were used to determine the lower limits of sensitivity for the SNaPshot technique in identifying SNPs. It was found that SNPs can be detected in urine spiked at cell concentrations of 10^8 cells/mL after incubation for 4hr to allow for active penetration and replication of *N. gonorrhoeae* within epithelial cells. If urine cultures are left for up to 24 hr, detection may be observed at *N. gonorrhoeae* concentrations of 10^7 cells/mL. However, many more replicates would be needed to prove this accurate. Using 5-

fold or even 2-fold dilutions in cell concentrations instead of 10-fold dilutions may have produced more of a gradient in product detection as seen in Figure 21. It is believed that cultures incubated for 4hr produce optimal amplicon to be used for the SNaPshot protocol rather than having longer incubations such as at 24hr (Figure 21). While there were slight differences between 10^7 cells/mL and 10^8 cells/mL cultures at 24hr, due to what we think of as oversaturation of DNA for a PCR, we did not test this hypothesis. However, if we had taken DNA isolated from the 10^8 cells/mL culture and performed 1:10 dilutions on these DNA preps, we may have been able to clarify if the limit of detection was due to problems associated with extraction or due to needing 10^8 cells/mL for detection.

This technique is also specific for *N. gonorrhoeae* SNPs. Cultures of *Staphylococcus aureus*, *E. coli*, *Lactococcus lactis, Lactobacillus acidophilus*, and *Moraxella osloensis* were tested using this technique. The reasons for testing with these organisms are as follows: *S. aureus* is a Gram positive bacteria isolated in many urinary tract infections (2), *E. coli* is the most commonly associated pathogen with urinary tract infections (10, 55), *L. lactis* can survive in the human gastrointestinal tract and can cross-contaminate vaginal secretions (59), Lactobacilli have been recognized as the predominant microflora of the human vagina (107), and *Moraxella sp.* are phylogenetically similar to Neisseria based on 16S ribosomal DNA (38). Within clinical samples of urine a mixture of these commensal and pathogenic species may be present (15). Thus, we wanted to ascertain that our technique was specific to only 1 species: *N. gonorrhoeae*. Ultimately, our *N. gonorrhoeae* oligonucleotides did not detect SNPs in any of these bacterial species

using SNaPshot and capillary electrophoresis most likely due to the specificity of our primer design. While this would indicate that this test can detect *N. gonorrhoeae* isolates, this method is not designed for this purpose. It was designed solely for the detection of SNPs within *N. gonorrhoeae* used as a model organism. It is believed that this technology could be applied to detecting antimicrobial resistant SNPs or other target SNPs in several bacterial species.

The versatility of the SNaPshot technique can be seen by its application within the field of microarray technology. The 16 to 50 bp oligonucleotide primers used in this study can also be used as probes for spotting onto microarray slides (15, 86). Thus, ssDNA products can be isolated from desired bacterial strains, labelled with a fluorescent probe and hybridized to the oligonucleotide to detect SNPs. Compared to antimicrobial susceptibility testing and DNA sequencing, the gold standard when it comes to identifying SNPs, this technique has a few limitations. For instance, SNaPshot can only identify mutations that have an oligonucleotide designed towards that particular SNP. It can not identify novel mutations involved in conferring resistance. Also, it can not accurately predict MIC values based on the mutation patterns seen in *gyrA*, *parC* and *mtr* although the correlation between the SNPs identified and MICs is strong. Molecular methods such as SNaPshot could not be used alone to predict quinolone resistance unless all other contributing genetic changes are identified. Therefore, it is advisable that diagnostic and public health laboratories do not abandon the capability to culture *N. gonorrhoeae* to measure antimicrobial resistance until a connection between all genetic determinants of resistance are identified (63). Otherwise, important

phenotypic surveillance data to monitor national changes in MIC values of susceptible strains will be absent. Although, at this point in time, this molecular technique could be utilized as a quick screening method for laboratories interested in characterizing the resistance profiles of resistant strains. A drawback may involve not many laboratories having access to the equipment needed to satisfy the protocol.

However, the numerous advantages of the SNaPshot method, outweigh the limitations when results are required urgently. For instance, this technique can be applicable to situations such as screening for ciprofloxacin resistance in *Bacillus anthracis* during a bioterrorism event. In such a situation, it is likely that one strain is used and it does not require testing all isolates. Additionally, the possibility of multiplexing many SNPs at one time is one of the main benefits. Multiplexing keeps the cost of analysis low since less reagents are used for each reaction. In addition, most genetic analyzers have the capacity to analyze up to 96 samples simultaneously with the majority of the steps in the procedure being automated. The entire process from start to results, including DNA isolation from urine samples, initial PCR amplification, SNaPshot PCR amplification, and capillary electrophoresis can be completed within 7.5 hr. Consistency between SNPs identified by SNaPshot compared to DNA sequencing was 100% for all samples tested.

In conclusion, the method we've presented is relatively simple and easily implemented in any laboratory with access to a thermocycler and a genetic analyzer. Continued focus and importance of SNPs is greatly improving, with the result of

increasing the cost-effectiveness of techniques that analyze them and its use within diagnostic and surveillance laboratories (71, 76).

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6. Appendix A

Antibiotic susceptibility of all *N. gonorrhoeae* isolates used in this study.

Strain	PEN	SPC	TET	ERY	AZI	CFX	CTX	CIP
ATCC49226	1	32	1	2	0.5	0.016	0.016	0.004
CDC10328	32	16	1	0.064	0.032	0.004	0.002	0.5
CDC10329	64	16	4	2	0.5	0.016	0.016	2
FA1090	0.125	32	0.5	4	4	0.002	0.001	0.004
I-20	0.064	16	0.25	0.25	0.032	0.016	0.002	0.004
I-21	0.5	16	1	0.5	0.125	0.016	0.016	0.004
I-22	0.016	16	0.25	0.5	0.125	0.001	0.0005	0.004
SPL4	4	16	4	2	0.25	0.25	0.125	16
18475	2	16	4	2	N/A	0.032	0.032	4
18610	2	16	2	1	0.125	0.016	0.016	16
18853	1	16	1	2	0.25	0.008	0.008	1
18907	2	16	4	2	0.5	0.016	0.016	16
19081	1	16	2	4	0.5	0.008	0.008	2
19132	1	16	2	2	0.25	0.016	0.016	2
19145	32	16	4	1	0.25	0.008	0.016	4
19255	1	16	4	2	2	0.032	0.016	0.008
19280	2	32	4	4	1	0.016	0.032	16
19283	1	32	4	4	2	0.064	0.032	0.016
19286	2	64	4	4	1	0.125	0.032	8
19289	4	32	8	4	1	0.032	0.032	8
19316	2	32	4	4	0.5	0.016	0.064	1
19324	2	32	4	4	0.5	0.016	0.032	16
19328	4	32	8	4	2	0.016	0.064	32
19330	128	32	4	2	0.5	0.008	0.016	8
19333	4	16	4	4	0.5	0.032	0.032	8
19378	2	32	4	4	2	0.032	0.032	0.016
19416	8	32	8	8	2	0.016	0.032	0.016
19491	2	32	8	16	2	0.032	0.032	16
19677	0.5	32	1	2	0.5	0.016	0.008	2
19703	2	32	4	4	1	0.016	0.016	16
19707	2	32	4	4	0.5	0.016	0.016	16
19709	4	32	4	4	1	0.032	0.064	16
19986	0.5	16	1	2	0.5	0.016	0.064	2
20419	32	32	1	0.5	0.125	0.016	0.008	2
20423	64	32	4	2	0.5	0.125	0.016	8
21145	2	32	4	2	0.5	0.032	0.032	8
21146	1	32	1	2	0.5	0.008	0.008	4
21631	0.5	32	8	8	2	0.008	0.016	0.008
23536	1	16	64	1	0.25	0.008	0.008	0.5
23895	32	16	0.5	0.064	0.032	0.004	0.002	0.064
23937	2	32	2	2	0.25	0.008	0.016	0.25
23953	128	32	64	2	0.25	0.016	0.016	0.25
24058	1	32	2	1	0.25	0.016	0.032	0.25
24059	1	32	2	1	0.25	0.016	0.032	0.25
24078	128	16	128	1	0.125	0.008	0.016	0.5
24118	64	16	64	1	0.125	0.008	0.008	0.25
24146	8	32	4	2	0.5	0.032	0.032	0.25
24170	2	16	32	0.0125	0.064	0.004	0.002	0.25
24178	8	32	4	2	0.25	0.016	0.016	0.25
24179	16	16	4	2	0.5	0.032	0.032	0.25
24228	1	32	2	2	0.25	0.016	0.016	0.5

24364	1	16	1	1	0.125	0.016	0.032	0.5
24380	1	16	4	2	0.125	0.016	0.032	0.5
24396	1	32	4	2	0.5	0.016	0.016	0.008
24425	0.032	16	32	0.125	0.064	0.008	0.002	0.004
24437	0.5	16	128	0.5	0.125	0.008	0.008	0.5
24489	1	32	4	2	0.25	0.008	0.016	0.5
24713	0.25	32	4	4	1	0.032	0.016	0.25
24795	0.064	8	0.064	0.064	0.008	0.004	0.001	0.064
24939	1	32	2	1	0.25	0.032	0.016	0.064
24967	1	32	4	2	0.5	0.032	0.016	0.032
24974	1	32	4	4	1	0.032	0.016	0.032
25092	0.5	16	64	1	0.125	0.004	0.008	0.5
25182	1	16	32	0.25	0.064	0.004	0.002	0.5
25255	1	16	32	0.25	0.064	0.008	0.004	0.5
25450	2	16	2	1	0.125	0.016	0.016	0.125
25713	1	32	2	2	0.5	0.032	0.016	0.008
25714	2	16	32	0.5	0.25	0.032	0.032	0.004
25716	1	16	2	0.5	0.25	0.032	0.016	0.008
25717	1	32	2	1	0.25	0.032	0.032	0.125
25718	2	16	2	0.5	0.25	0.016	0.016	0.008
25719	1	16	2	0.5	0.25	0.016	0.016	0.008
25722	2	32	4	2	0.5	0.032	0.016	0.032
25727	0.125	16	32	0.25	0.064	0.004	0.002	0.004
25729	2	32	4	2	0.25	0.032	0.032	0.016
25733	2	32	4	2	0.5	0.016	0.016	0.016
25736	1	16	2	0.5	0.125	0.032	0.032	0.125
25737	1	16	2	1	0.125	0.032	0.032	0.125
25742	2	32	4	2	0.5	0.016	0.016	0.016

PEN - penicillin, SPC -spectinomycin, TET - tetracycline, ERY - erythromycin, AZI - azithromycin, CTX - ceftriaxone, CFX – cefixime, and CIP – ciprofloxacin.