DEVELOPING RAPID MOLECULAR TECHNIQUES FOR DIAGNOSIS OF *NEISSERIA GONORRHOEAE* AND CHARACTERIZATION OF ANTIMICROBIAL RESISTANCE

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ABSTRACT

Multidrug-resistant *Neisseria gonorrhoeae* (Ng) is a global health emergency. To stop the spread of untreatable gonorrhea, improved molecular diagnostics and characterization of antimicrobial resistance (AMR) determinants are crucial. For this research, I developed an RT-PCR based molecular diagnostic test to identify Ng as well as a second test, utilizing novel multiplex primers for *gyrA*, to simultaneously identify Ng and predict ciprofloxacin susceptibility. Although ciprofloxacin is no longer recommended for the treatment of gonorrhea, susceptible isolates can be prevalent in some regions. Thus, if the ciprofloxacin susceptibility of an isolate is known, this antibiotic could be used for treatment. Both the Ng diagnostic test and the test for ciprofloxacin susceptibility performed with high sensitivity and specificity with DNA from cultures and gave more variable results with clinical specimens (i.e. urines and remnant Aptima urine specimens). These tests were modified for the incorporation of the primers into a hydrogel-based diagnostic platform, for eventual point-of-care applications. The hydrogel platform performed with high sensitivity and specificity for Ng diagnosis, depending on the specimen type. Additionally, to identify emerging AMR genotypes, various genes implicated in Ng AMR resistance were amplified and sequenced from Ng positive remnant Aptima urine specimens (for which AMR testing is not possible). Phenotype predictions based on DNA sequence analysis indicated emerging azithromycin resistance in Saskatchewan and highlighted the importance of molecular AMR surveillance to prevent Ng outbreaks. Finally, the properties of a β-lactamase produced by an uncharacterized Ng plasmid was investigated. A novel 6bp deletion in the start codon of *bla*TEM was identified. Due to this deletion, the β-lactamase was truncated and resulted in slow ampicillin hydrolysis coupled with a low penicillin MIC (0.125 mg/L). Overall, my research produced molecular diagnostic tests for Ng and ciprofloxacin susceptibility prediction and characterized and predicted emerging AMR resistance by molecular analysis.

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LIST OF ABBREVIATIONS

AMR	Antimicrobial Resistance
AMS	Antimicrobial Susceptibility
CDC	Centers for Disease Control and Prevention
CE	Conformité Européenne (European Conformity)
CE-IVD	Conformité Européenne (European Conformity) – *In vitro* diagnostic device
CLSI	Clinical and Laboratory Standards Institute
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDA	Food and Drug Administration
MSM	Men Who Have Sex With Men
MIC	Minimum Inhibitory Concentration
NML	National Microbiology Laboratory
NG/Ng	*Neisseria gonorrhoeae*
NAAT	Nucleic Acid Amplification Tests
POCT	Point-of-Care Test
PCR	Polymerase Chain Reaction
RT-PCR	Real-Time Polymerase Chain Reaction
RRPL	Roy Romanow Provincial Laboratory
SHA	Saskatchewan Health Authority
SNP	Single Nucleotide Polymorphism
STBBI	Sexually Transmitted and Blood-Borne Infections
STI	Sexually Transmitted Infections
UN	United Nations
UNICEF	United Nations Children's Fund
WGS	Whole Genome Sequencing
WHO	World Health Organization

CHAPTER 1: INTRODUCTION

1.1 *NEISSERIA GONORRHOEAE*

1.1.1 Gonorrhea Infection

Gonorrhea, a sexually transmitted infection (STI) caused by the pathogen *Neisseria gonorrhoeae* (the gonococcus), remains an important global public health concern with 86.9 million new infections worldwide each year (1). Gonococci, Gram-negative diplococci belonging to the Neisseriaceae family, are human restricted and are believed to have co-evolved with their human host for centuries (2). *N. gonorrhoeae* and its closely related opportunistic pathogen, *Neisseria meningitides*, are considered to have evolved from commensal *Neisseria* species in the pharynx (3). In heterosexuals, *N. gonorrhoeae* primarily causes urogenital tract infections resulting in urethritis and cervicitis in women and urethritis in men (4, 5). However, with differing sexual practices and norms, especially in men who have sex with men (MSM), gonococcal infections are more commonly reported from extragenital sites (i.e. rectum and pharynx; 2). Gonorrhea infections in the genital tract are largely symptomatic in men (at least 90%) but are asymptomatic or present with non-specific symptoms in women during the primary stages of the infection. As a result, gonococcal infections in many women are undiagnosed. If left untreated, ascending gonococcal infections can lead to severe reproductive complications such as salpingitis which in turn can result in pelvic inflammatory disease, ectopic pregnancy, complicated pregnancy, and infertility (2). Additionally, untreated infections can cause epididymitis in men. Gonococcal infections can also lead to the acquisition of other STIs such as Chlamydia, Mycoplasma and the Human Immunodeficiency Virus (HIV; 2). Furthermore, pregnant women with *N. gonorrhoeae* can transmit the infection to infants during birth resulting in ophthalmia neonatorum, a leading cause of blindness in newborns before the introduction of antibiotics (2).

Despite the number of cases reported worldwide, there is still no vaccine to prevent infections caused by *N. gonorrhoeae*. One limitation is the lack of an ethically acceptable animal model of challenge. The only functional mouse model to date is female mice treated with 17β-estradiol (6) and transgenic mouse models expressing human receptors are currently under study (7, 8). Human challenge models have been conducted in men to study the early stages of colonization. This model

has limitations in that treatment has to be administered at the onset of first symptoms and testing is not permitted in women due to the higher risk of complications (9). Even though primates (i.e. chimpanzees) can be used as a challenge model, costs and ethical constraints of using larger animals hinders the use of this model. Another reason is that *N. gonorrhoeae* is antigenically highly variable and only two antigens (i.e. purified pilin and killed whole cells) have been tested in clinical trials, with no success (10). Third, and owing to antigenic variability, despite repeat infections correlates of protection have not been identified in humans (10). Without a vaccine, prevention largely relies on testing, especially at the onset of symptoms, partner notification, epidemiological surveillance, and education of safer sexual practices. Treatment relies on effective antibiotic therapy, where the current standard in North America is co-treatment with 1g azithromycin orally and 250 mg ceftriaxone intramuscularly. In the absence of ceftriaxone, 1g Azithromycin orally is recommended with 2g spectinomycin intramuscularly (11). In general, Canada, the United States, Europe, Australia and Brazil recommend dual treatment with ceftriaxone and azithromycin at differing concentrations for gonococcal infections. Brazil is the only nation that still recommends ciprofloxacin in combination with azithromycin, primarily due to lower resistance levels to ciprofloxacin (12). Strikingly, the United Kingdom's 2019 STI treatment guidelines no longer recommend azithromycin as the first-line treatment of gonorrhea due to the alarmingly high levels of azithromycin resistance reported in Europe (13). Rather, the recommended treatment is mono-therapy with 1g ceftriaxone or ciprofloxacin for susceptible infections (13).

1.1.2 Prevalence of *N. gonorrhoeae*

In an era of rising gonorrhea infections, a 67% increase in the number of infections was observed in the United States from 2013 to 2017. Similarly, an increase in the number of infections in MSM, at 38.5%, was noted in 2017 (14). Gonorrhea statistics from the United States date back to 1919 (15). The rates are even more striking in Europe, where an over 200% increase was observed from 2008 to 2017 (2). In Europe, 30% of all reported infections in 2017 stemmed from MSM (14). Diagnosis and treatment for gonorrhea have been provided in England and Wales since 1916 (15). Gonorrhea has been a notifiable disease in Canada since 1924. In 2017, 29,034 gonorrhea infections were reported in Canada, corresponding to a 109% increase from the reported rates in 2008 (Figure 1.1; 17). From reported gonorrhea infections in 2017, 65% was males and 35% was

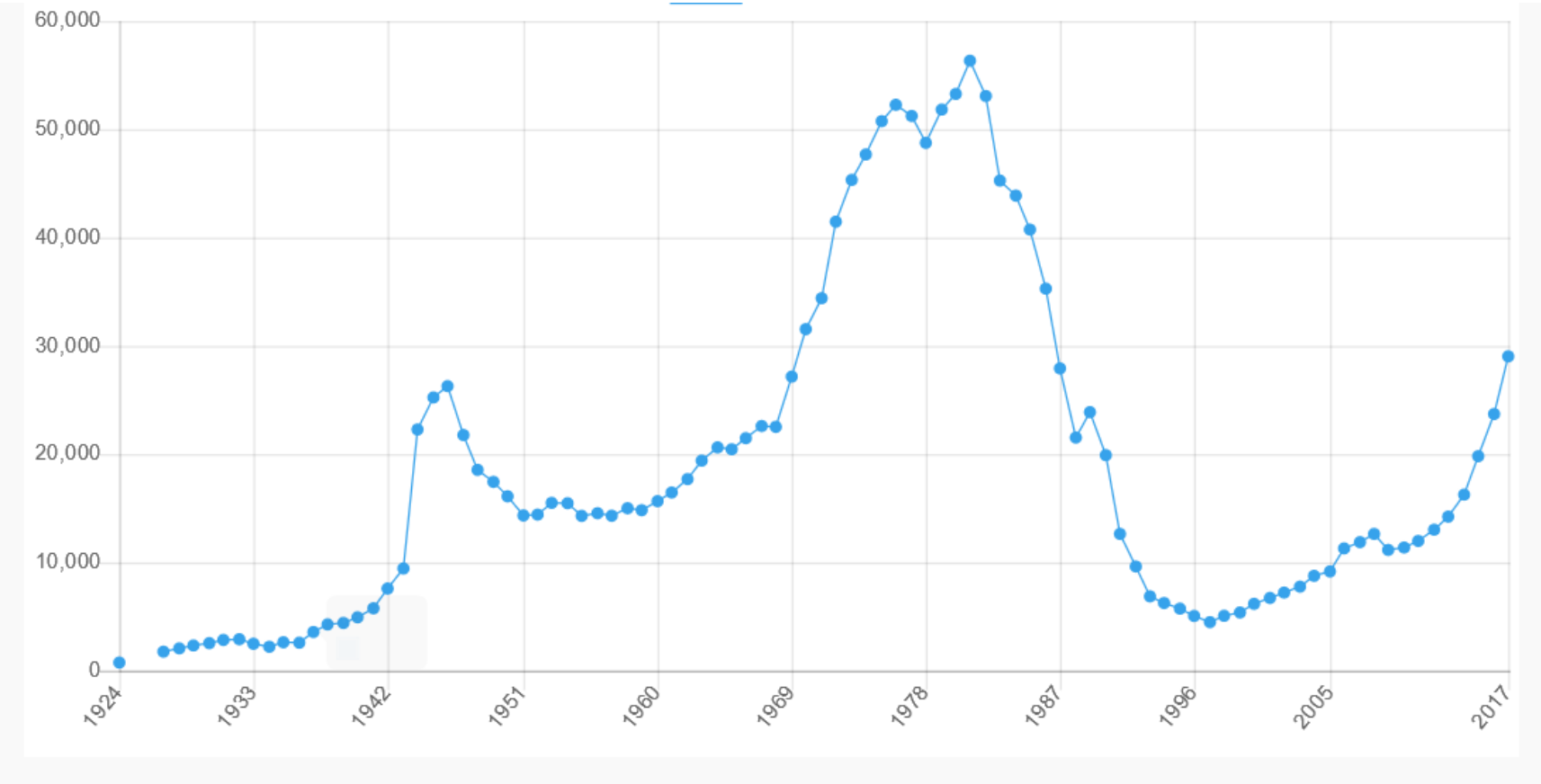

Figure 1.1 Number of reported gonorrhea infections in Canada from 1924 to 2017. Figure adapted from the 'Reported cases from 1924 to 2017 in Canada – Notifiable diseases online' 2019. Public Health Agency of Canada. https://diseases.canada.ca/ndis/charts.php?c=pl.

female (16). The highest rate of infection was reported among 20-24-year-olds. Notably, the number of cases reported in men increased with age and the highest rate was reported among men over 60 years. Conversely, the number of cases reported decreased among older women (16). The rates of gonorrhea vary between Canadian provinces and territories. The highest rates in 2017 were reported from Nunavut and Northwest Territories (over 1,000 cases per 100,000 population), while the largest increase (610%) since 2013 was noted in the Yukon (Figure 1.2). The lowest rates were reported from the Maritime Provinces, followed by Ontario, British Columbia and Quebec (less than 100 cases per 100,000 population). Alberta, Saskatchewan and Manitoba observed high numbers of cases at 112, 193 and 250 cases per 100,000 population respectively (16).

1.1.3 Costs Associated with *N. gonorrhoeae* Infection

The economic burden of *N. gonorrhoeae* is striking in terms of primary health care costs, diagnosis, hospitalizations, long-term disabilities, and lost productivity. This cost would be substantially higher in the absence of proper diagnostic measures (17, 18). The estimated cost in Canada for hospital care for gonorrhea is CAD $247 per person per visit (19). However, detailed information on the economic burden of gonorrhea in Canada is lacking. In the United States, the lifetime direct medical costs for gonorrhea was USD $162.1 million (18). The estimated cost per single case of gonorrhea was USD $354 for women and USD $79 for men (20). When costs were assessed for diagnosis and treatment, the pelvic inflammatory disease was USD $3202 per individual while the cost for epididymitis in men was USD $313 per individual (20). It is important to note that the total costs reported here do not include costs associated with gonorrhea prevention measures, lost productivity and costs associated with personal suffering. Other estimates of productivity loss due to STIs have indicated that these costs are equal or in some cases significantly higher than the associated direct medical costs (18).

As per estimates by the United Nations Children's Fund (UNICEF), the costs of therapeutics and service delivery combined, average USD $10.71 for treatment of gonorrhea per person (21). In Saskatchewan, the combined cost for diagnosis of chlamydia and gonorrhea was CAD $31.50 including specimen collection (CAD $10.50) and testing (CAD $21) (unpublished information from Roy Romanow Provincial Laboratory, Regina, SK). The government of Alberta has

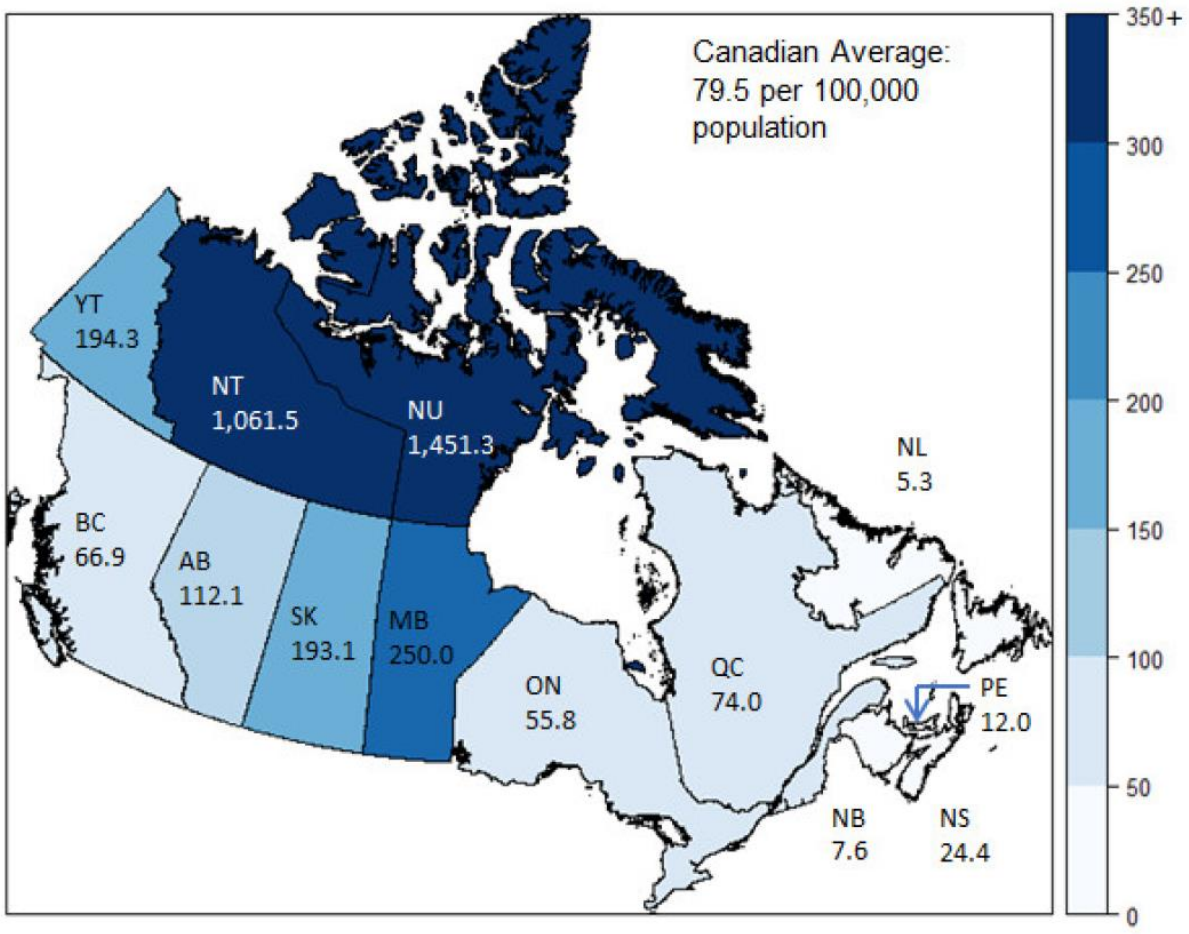

Figure 1.2 Rates of reported gonorrhea infections in Canadian provinces and territories in 2017. Figure adapted from the 'Report on sexually transmitted infections in Canada', 2017. Public Health Agency of Canada.

estimated that for every dollar spent on early detection and timely treatment of gonorrhea and chlamydia, CAD $12 could be saved in the long term costs of delayed or non-treatment (22).

A recent survey estimated the costs associated with the implementation of a national STI program, as part of the Global STI control strategy from 2016-2021. Here, it was found that the implementation itself will cost USD $18.1 billion in low and middle-income countries (21). The associated costs for screening are USD $3.69 billion, while a staggering USD $3 billion is associated with the clinical management of urethral discharges, vaginal discharges and genital ulcers alone (21). Embedded in this cost is USD $1.4 billion for chlamydia and gonorrhea testing and another USD $818 million for service delivery. It is projected that the global costs of treating gonorrhea and chlamydia will increase from USD $2.6 billion to over USD $4 billion within this 5-year time frame. The 5-year cost associated with screening for gonorrhea and chlamydia combined is projected at USD $463,132,876 for low and middle-income countries (21). Thus, a cost-effective test for *N. gonorrhoeae* diagnosis would reduce the burden on the health care system and allow the implementation in resource-constrained settings.

1.2 ANTIMICROBIAL RESISTANCE

1.2.1 Overview of Antibiotics for Treating *N. gonorrhoeae* Infections

Antimicrobial resistance (AMR) has been identified as a foremost threat to global health by various health agencies including the United States Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO). In September 2016, the United Nations (UN) General Assembly held a meeting of global leaders to engage them in the fight against AMR and to implement the Global action plan on AMR. It was only the 4th time in history that a health topic was addressed by the UN General Assembly. The problem of AMR is so severe that antibiotics may become ineffective within the next few decades (25). Indeed, the emergence of multidrug-resistant, untreatable bacteria has already been described foreshadowing a post-antibiotic world. The rise in gonorrhea infections is partially attributed to the emergence of isolates with decreased susceptibility and resistance to many of the first-line antibiotics used for treatment (26). It was reported that 63% of Canadian isolates tested in 2017 were resistant to at least one antibiotic, where 11.7%, 0.6% and 0.5% were resistant to azithromycin, cefixime and ceftriaxone respectively (27).

Extended-spectrum cephalosporins (ESC) cefixime and ceftriaxone were discovered in the latter part of the 1940s and introduced for gonorrhea treatment in the early 1980s. The use of ceftriaxone increased in response to ciprofloxacin resistance and the withdrawal of this antibiotic from treatment guidelines (25, 26). Cefixime was introduced for gonorrhea treatment prior to ceftriaxone; however reduced susceptibility was quick to emerge (11) and treatment failures were reported in Europe in the early 2000s (27-29) and have continued to emerge in North America, South Africa, Japan, Australia, Sweden, and Slovenia (30, 31).

Of concern is that resistance was also reported to ceftriaxone, the last antibiotic recommended for monotherapy. The first ceftriaxone resistant isolate was identified in Japan in early 2000, and two years later resistance was also reported from Spain and France. Shortly after reports of ceftriaxone resistant isolates began to emerge from China, Argentina, and Singapore and treatment failures were reported from Australia, Japan and several European countries (32-35). Two instances of ceftriaxone resistant *N. gonorrhoeae* have been reported in Canada from 2017-2018 (36, 37). Despite the reported treatment failures, ceftriaxone remains the last antibiotic recommended for monotherapy of gonorrhea (30, 31).

Azithromycin, a macrolide, was first introduced for gonorrhea treatment in the 1980s (38). The first resistant isolates were from Latin America in the 1990s (38, 39) and the first report of high-level azithromycin resistance was reported in Argentina (40) These reports were soon followed by resistance reports of azithromycin from the Latin America, United States, China, Canada, Scotland and Italy, where between 2014 and 2015 even isolates with azithromycin MIC over 256 g/mL were reported from England (Figure 1.3; 41-44). Regina, Saskatchewan has also observed over 5% azithromycin resistant gonococcal isolates in 2010. However, the overall prevalence of azithromycin resistance in the province, and in Canada, was well below the WHO recommended 5% cut-off limit (45); which states if more than 5% of isolates tested are resistant to a given antibiotic, the antibiotic should be discontinued from treatment (45). 2014 marked the first report of dual treatment failure (azithromycin and ceftriaxone) in the United Kingdom (46). Additionally, isolates with high-level azithromycin and ceftriaxone resistance were also isolated from Australia

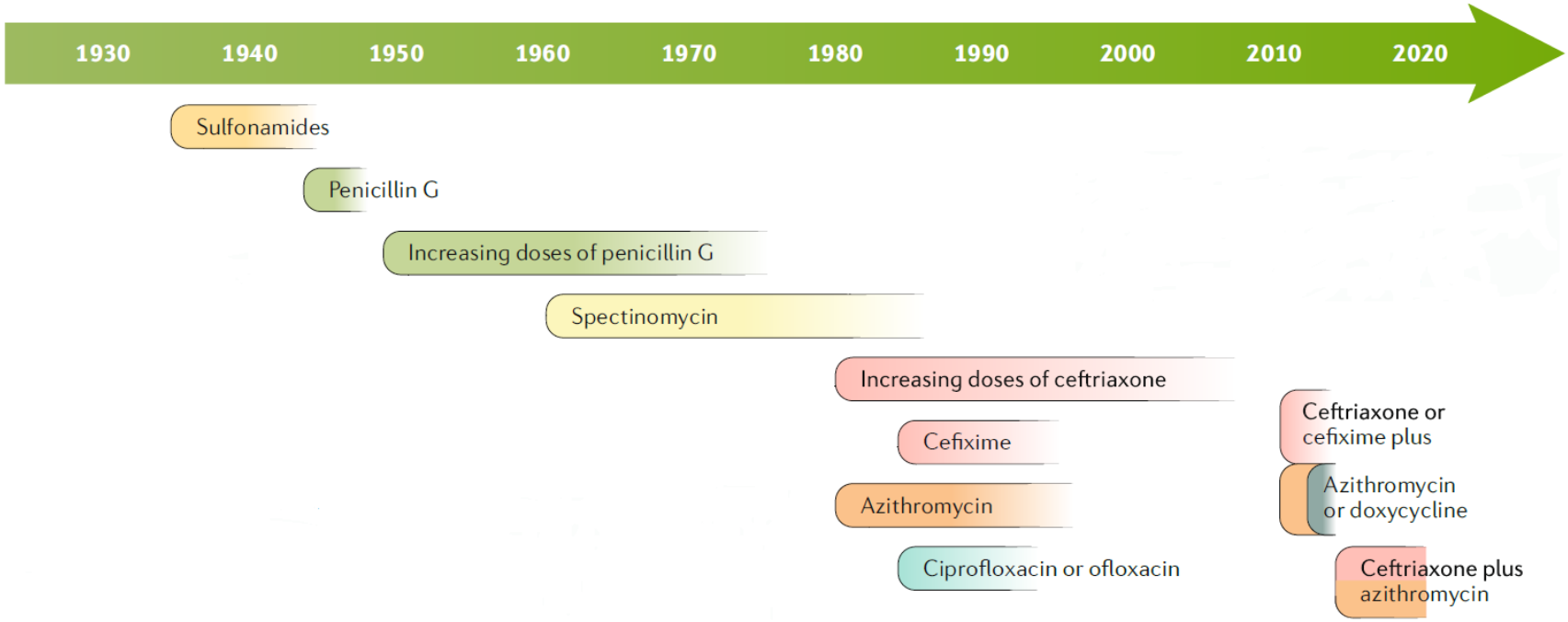

Figure 1.3 Emergence of gonococcal resistance to various antibiotics for nearly 90 years. The length of the bar represents the time since the introduction of the antibiotic to the emergence of resistance to the given antibiotic. Figure adapted with modifications from Unemo et al., 2019. Gonorrhoea. *Nat Rev Dis Primers*. 5:79. The figure is reproduced with permission from *Nat Rev Dis Primers*.

and the United Kingdom (35, 47, 48). Consequently, azithromycin is no longer recommended as a first-line treatment for gonorrhea in the United Kingdom (13). Highlighting the graveness of this finding, a ceftriaxone resistant strain from Japan has been reported in many other countries by 2015 (49-51).

Ciprofloxacin, a fluoroquinolone antibiotic, was introduced in the late 1980s to replace tetracycline due to high levels of tetracycline resistance (52). Despite the observance of reduced susceptibility and treatment failures in 1989 and the early 1990s, ciprofloxacin was prescribed as a monotherapy for almost two decades and was removed from recommendations in the United States, Canada, Europe and Asia only in mid-2000s (38, 53-55). However, isolated communities with ciprofloxacin susceptible isolates in Canada, the United States, Europe and Australia hold promise in re-introducing this antibiotic for treatment (45, 56-58).

First introduced in 1943, resistance to penicillin in *N. gonorrhoeae* was observed in the early 1950s but was prescribed until the 1980s at increasingly higher concentrations (15, 59, 60). In 1950s tetracycline was introduced for the treatment of penicillin-allergic individuals (61). However, by 1985 high-level tetracycline resistance was observed in the United States and was no longer recommended for treatment (61, 62). Sulfonamides, the first antibiotic for gonococcal treatment, was introduced in the 1930s, and less than 15 years later more than 75% of treatment failures were reported (Figure 1.3; 63, 64). Combined sulfonamides and trimethoprim treatment was used until the 1970s (65, 66). Even at least one documented gonorrhea treatment failure with doxycycline was reported from France in 2017 (67).

1.2.2 Antibiotic Resistance Mechanisms

Drug resistance in *N. gonorrhoeae* arises through a multitude of mechanisms including both chromosomal and plasmid-mediated. Remarkably, the accumulation of resistance determinants does not affect gonococcal fitness (2). Resistance to ESCs can arise through mutations in *penA* (PBP2), *penB* (PorB), *ponA* (PBP1) and/or *mtrR* (MtrR). Furthermore, it is speculated that there remain unknown mechanisms of ESC resistance and are termed ‘factor X’ (25, 26). Cefixime resistance is primarily driven by substitutions in *penA* (A501V, I312M, V316T, G545S, A501V

and N512Y), while *porB* (G120K/A121N/S, G120K/A121G) and *mtrR* (A-, A38T, G45D) alterations also play minor roles (11).

As ceftriaxone binds PBP2 with high affinity, *penA* remains the main resistant determinant (G542S and P551S/L) for ceftriaxone (11). Accumulation of mutations in *penA* alters the structure of PBP2 and prevents ceftriaxone binding (Figure 1.4). *penA* mutations arise through single nucleotide polymorphisms (SNPs) and genetic recombination between *N. gonorrhoeae* and other commensal *Neisseria* species, which result in mosaic *penA* alleles (11, 25, 38, 68). Compared to wild-type, mosaic *penA* alleles encoding PBP2 can contain up to 60 amino acid changes. Two of the well known mosaic *penA* alleles implicated in ESC resistance is *penA* type X with A311V, A328T, T316P and T484S substitutions, and *penA* type XXXIV with A501P substitution (34, 69, 70). Highlighting the gravity of emerging ceftriaxone resistance, recently a Canadian report identified a ceftriaxone resistant FC428 *N. gonorrhoeae* clone, which was originally identified in Japan (71). Additionally, ceftriaxone resistance clones F90, GK124 and IR72 have been reported from France, Denmark and Ireland respectively (61, 72, 73).

Azithromycin works by preventing the elongation of the peptidyl transferase polypeptide chain within the ribosomal subunit (74, 75) and resistance arises through several mechanisms (Figure 1.4). Primarily resistance is driven by mutations in 23S rRNA, where A2059G and C2599T substitutions in 1-4 alleles encoding 23S rRNA result in high-level resistance, while C2611T substitution results in low-level resistance (11, 76). Although mutations in single alleles do not have a profound effect on azithromycin MICs, high-level resistance was reported when these substitutions occur in all four alleles (77-80). Furthermore, low-level resistance to azithromycin is mediated by rRNA methylases (*ermB*, *ermC* and *ermF*) that methylate a single adenine at position 2058 of the 23S rRNA peptidyl transferase domain, preventing macrolide interaction with the ribosome (38, 77, 81). Additionally, overexpression of the multiple transferable resistance efflux pump (MtrCDE) through mutations in *mtrR* (A-, A38T, and G45D) repressor can elevate azithromycin MIC to 0.5 g/mL (82, 83), and mutations in the L4 ribosomal protein, which comes in to contact with the peptidyl transferase of 23S rRNA, can influence the RNA confirmation (83-86). Although not common, overexpression of MacAB efflux pump and *mef* encoded efflux pump can also decrease susceptibility by exporting azithromycin out of the cell (38, 74).

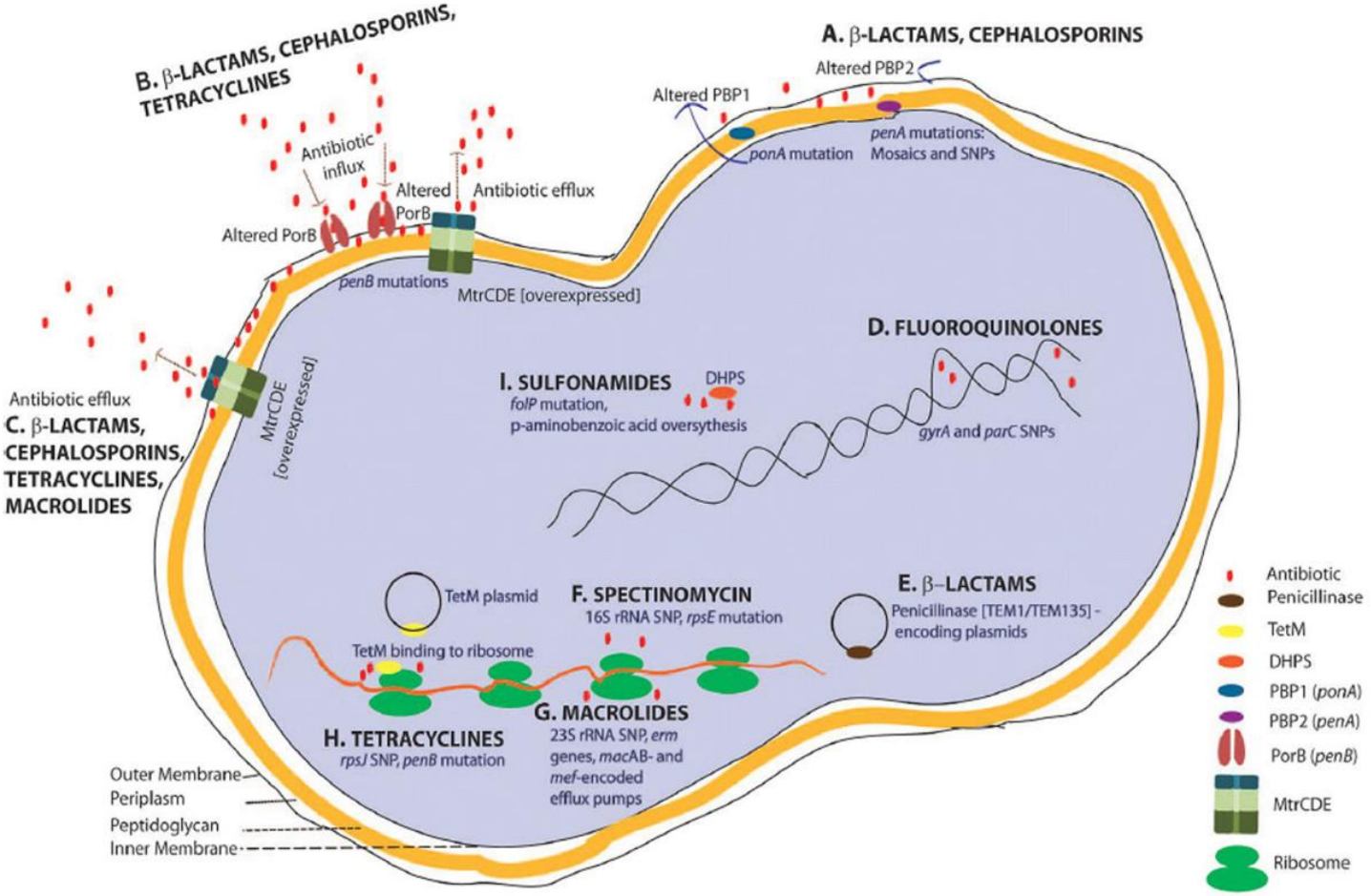

Figure 1.4 Antibiotics and their respective resistance mechanisms in the gonococcus. Figure adapted from Dillon et al., 2015. *Culture*. 35:1. The figure is reproduced with permission from Thermo Scientific.

Ciprofloxacin influences DNA gyrase and topoisomerase IV activity, which affect DNA replication, recombination, transcription and repair mechanisms (11). Ciprofloxacin resistance in gonococci is mediated through substitutions in the quinolone resistance determining region (QRDR), namely within the nucleotide sequences of DNA gyrase A (*gyrA*) and topoisomerase IV (*parC*; 87). Substitutions in GyrA S91 (S91F/Y), A92 (A92P) and D95 (D91A/G/N) positions alone are associated with resistance (88-90). However, combined GyrA and ParC (D86N, S87R/N, S88P, and E91K) substitutions lead to ciprofloxacin MIC over 32 g/mL (74, 91, 92).

Tetracycline binds to the bacterial 30S ribosomal subunit and prevents protein synthesis (93, 94). Similar to penicillin resistance, *porB* (G120K/A121N, A121S, G120N/A121G) and *mtrR* (A-, A38T, G45D) mutations also produce tetracycline resistance (59, 95). An additional substitution (V57M) in the 10S ribosomal protein (*rpsJ*) can elevate tetracycline MICs to over 2 g/mL (Figure 1.4; 88, 104). In addition to chromosomal resistance, TetM (*tetM*) produced by conjugative plasmids prevents tetracycline from binding to the ribosome and leads to high-level tetracycline resistance (38, 59, 61).

Penicillin works in the periplasm and prevents bacterial cell wall synthesis by binding to penicillin-binding proteins (PBP; transpeptidase enzymes). Resistance to penicillin occurs through mutations in *penA*, *ponA*, *penB*, and *mtrR*. Here *penB* (porin protein B; PorB) and *mtrR* (multiple transferable resistance transcriptional repressor of MtrCDE efflux pump; MtrR) works by regulating the antibiotic concentration in the cell, while *penA* (penicillin-binding protein 2; PBP2) and *ponA* (penicillin-binding protein 1; PBP1) works by altering the PBP structure (Figure 1.4; 74, 97).

Acquisition of mutations in *penA*, *mtrR*, *porB* and *ponA* have been associated with stepwise increases in MICs to penicillin. The 345D insertion in PBP2, along with an additional 4 to 8 substitutions (i.e. F504L, A510V, A516G, H541N, P552V, K555Q, I556V, and I557V) in *penA* lead to an intermediate MIC to penicillin (95, 98, 99). The L421P substitution in PBP1 decreases the acylation with the β-lactam structure in β-lactam antibiotics, while G120K/N and A121D/G/N substitutions in PorB are associated with elevated MICs to penicillin (11, 100, 101). Importantly, PorB substitutions and elevated MICs to penicillin were noted only when the MtrCDE efflux pump was also overexpressed (102). Overexpression of MtrR elevates MICs not only of penicillin but

also of tetracycline and macrolides (103, 104). This is achieved through a single nucleotide deletion (A-) or a dinucleotide insertion (AA) in the *mtrR* promoter and A38T or G45D substitutions in MtrR (82, 104, 105).

Chromosomally mediated resistance mechanisms lead to penicillin MICs of up to 4 mg/L. In the 1970s, penicillin MIC up to 128 g/mL were observed (34, 106) and were attributed to a plasmid-mediated β-lactamases. Gonococcal isolates with β-lactamase producing plasmids are known as penicillinase-producing *N. gonorrhoeae* (PPNG; 104, 105). Over the years, seven genetically related β-lactamase producing plasmids with various insertions and deletions have been identified in *N. gonorrhoeae* and these plasmids were named according to their geographical origin, namely Asia, Africa, Toronto/Rio, Nimes, New Zealand, Johannesburg and Australia (106-109).

Sulfonamides act by competing for dihydropteroate synthetase (DHPS), an enzyme in the folic acid biosynthesis pathway (Figure 1.4). Gonococci have developed mechanisms to overproduce p-aminobenzoic acid, the substrate for DHPS to develop sulfonamide resistance, or to mutate *folP* (dihydropteroate synthase) to synthesize a mutated DHPS that has low affinity to sulfonamides (59). Similar to sulfonamides, trimethoprim works on the folic acid synthesis pathway by inhibiting the activity of dihydrofolate reductase (DHFR). However, *N. gonorrhoeae* is less sensitive to this antibiotic because gonococcal DHFR has a low affinity for trimethoprim (59, 110, 111).

1.3 *N. GONORRHOEAE* SURVEILLANCE

1.3.1 Typing Methods

Isolate tracking by using strain differentiation techniques is vital to identify the clonal spread of *N. gonorrhoeae* in a population (112). In response, over the years several typing schemes for *N. gonorrhoeae*, from phenotypic to next-generation sequencing, have emerged (113). Typing increases gonococcal surveillance and epidemiological awareness, traces the evolutionary history and measures population dynamics of the pathogen, while providing contact tracing and rational treatment, and facilitates control and prevention initiatives (112, 114).

The *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) scheme (http://www.ng-mast.net/) is based on sequence analyses of two highly polymorphic genes, *porB* and *tbpB* (115). Upon sequence submission of *porB* and *tbpB*, sequence types are assigned separately to both of these genes and a final NG-MAST number is assigned to the isolate harbouring the given *porB*/*tbpB* sequencing pattern (115). NG-MAST analysis is useful if a higher discriminatory power between the isolates is required, i.e. to differentiate large *porB* based *N. gonorrhoeae* clusters (115).

In contrast to the NG-MAST, multilocus sequence typing (MLST) scheme for *N. gonorrhoeae* (https://pubmlst.org/neisseria/) is based on sequence analysis of a panel of conserved housekeeping genes in *N. gonorrhoeae* (eg. *porA*, *porB*, *fetA*, *fHbp*, *nadA*, and NHBA) and represents genetic variation in the *N. gonorrhoeae* population (112, 116). Due to the presence of conserved loci and their slow accumulation of mutations, these genes reflect the spread and the natural evolution of gonococci. However, MLST typing schemes are more suitable for the long term and large scale global epidemiological analyses, while NG-MAST is more suitable for analyzing short term and local outbreaks (114, 115).

Recently, a curated web-based sequence typing scheme, *N. gonorrhoeae* sequence typing for antimicrobial resistance (NG-STAR; https://ngstar.canada.ca/alleles/query?lang=en), was introduced (117). Using DNA sequences of seven known AMR determinants (*penA*, *mtrR*, *porB*, *ponA*, *gyrA*, *parC*, and 23S rRNA), this scheme identifies specific mutations leading to antibiotic resistance in *N. gonorrhoeae* isolates and assign sequence types (117). Based on the mutations, a MIC value for each antibiotic is also predicted.

Although almost every nucleic acid-based typing method is suitable for *N. gonorrhoeae* molecular surveillance, these analyses have been mostly limited to retrospective studies. Sequencing whole genomes from *N. gonorrhoeae* positive clinical specimens or cultures can simultaneously provide an in-depth analysis of the prevalence of AMR and strain types. Already a whole-genome sequencing (WGS) based platform, Gen2Epi (ftp://www.cs.usask.ca/pub/combi), integrating NG-MAST, MLST and NG-STAR, has been developed and tested with a large repository of *N. gonorrhoeae* genomes (118). The increase in WGS data allows robust AMR determination from

gonococcal isolates, especially because WGS has "superior resolution" in tracking disease transmission and outbreak spreading compared with conventional methods such as NG-MAST and MLST (119, 120). Furthermore, WGS has facilitated the monitoring of the AMR evolution in *N. gonorrhoeae* and the development of countermeasures to address this by identifying mutations leading to drug resistance in *N. gonorrhoeae* (121). Thus, the development of WGS has revolutionized the characterization of microbial isolates such as *N. gonorrhoeae* and has impacted typing methods, genetic analyses, transmission and epidemiological surveillance studies.

1.4 SCREENING AND DIAGNOSIS

1.4.1 Microscopy

Despite the availability of more advanced molecular tests, microscopic diagnosis is still used (Table 1.1). The hallmark of *N. gonorrhoeae* is Gram-negative diplococci within polymorphonuclear leucocytes under the field of a light microscope. However, the sensitivity and specificity of the Gram stain depend on the specimen type, where higher sensitivities are observed with urethral swab specimens from symptomatic males (122-125). Lower bacterial load from asymptomatic males and endocervical specimens, especially when a large number of other bacterial species are also present, makes it challenging for Gram stain-based diagnosis of gonorrhea. The Gram stain is not recommended for the diagnosis of rectal specimens due to low sensitivity (≤40%) and pharyngeal specimens due to the presence of commensal *Neisseria* species. Alternatively, methylene blue staining, instead of Gram staining, has also been used for the diagnosis of gonorrhea (124-127). In instances of obvious symptoms, (i.e. purulent discharge from the urethra) a Gram stain is sufficient to confirm *N. gonorrhoeae* infection. However, culturing accompanied by biochemical tests is required for asymptomatic genital infections and extragenital infections such as pharyngeal, rectal and ocular sites (2, 128).

1.4.2 Culture

For decades, culture has been the gold standard for gonococcal diagnosis and remains the only means of diagnosis in remote settings where more expensive molecular diagnostic methods are

Table 1.1 Comparison of diagnostic methods for *N. gonorrhoeae*. Table adapted from Unemo et al., 2019. Gonorrhoea. *Nat Rev Dis Primers*. 5:79. The table is reproduced with permission from *Nat Rev Dis Primers*.

Parameter	**Microscopy**	**Culture**	**NAAT**
Specimen types			
Urine	No	No	Yes[a]
Urethral swab	Yes	Yes	Yes
Rectal swab	No	Yes	Yes/no[b]
Pharyngeal swab	No	Yes	Yes/no[b]
Conjunctival swab	Yes	Yes	Yes/no[b]
Performance			
Sensitivity[c]	Low-high	Moderate-high	Very high
Specificity[c]	Moderate-high	Very high	Moderate-very high
Cost	Low	Moderate	Moderate-very high
Instrumentation	Microscope	Routine microbiology	Moderate-large footprint
Technical complexity	Low-moderate	Moderate	Low-high
Level of laboratory infrastructure	Low	Low-intermediate	Intermediate-high
Potential as a POCT	Yes	No	Yes

[a]Sensitivity of urine specimens can be lower than with other specimen types and a negative result does not exclude gonococcal infection.
[b]Not all platforms have received FDA approval for the given specimen.
[c]Sensitivity and specificity depend on the specimen type.

unaffordable. Furthermore, culture is required to perform subsequent biochemical characterization tests. Culture is still the method recommended for pelvic inflammatory disease, cases of sexual abuse, treatment failures and AMR testing (123-125, 129). Specimens for culture should be obtained at least two days post-exposure, as samples collected sooner may fail to grow. As with Gram staining, the sensitivity of culture depends on a combination of factors (Table 1.1). The method of sample collection and transportation is vital for culturing as the viability of the organism depends on sample handling and proper transportation. The viability of gonococci can be preserved up to 12h at room temperature when transport swab systems (with or without charcoal) are used (130). Depending on the size of the inoculation, viability may be prolonged. JEMBEC plates containing Martin-Lewis agar and a CO_2 generating tablet, as well as chocolate agar slants are also acceptable means of transportation (130).

The anatomical site of the specimen, growth media and incubation conditions also play a role in the growth of cultured specimens (Table 1.1; 124, 125, 129). Sensitivities of culture also depend on the expertise of the laboratory personnel. For example, sensitivities for urogenital specimens range from 72-95%, while the same specimen cultured by an experienced technician could give a 95-100% sensitivity (2, 124, 125). Upon arrival, the specimen should be inoculated on selective agar (i.e. Thayer-Martin, New York City, Martin Lewis medium) with antimicrobials (i.e. vancomycin, colistin, trimethoprim, nystatin, amphotericin and/or anisomycin) to inhibit the growth of commensal bacteria and fungi (130). Following inoculation, plates should be incubated at 35-37°C with 5% CO_2 in a humid environment for 18 to 24h (38). Subsequent to primary isolation on selective media, isolates should be subcultured on non-selective media (i.e. gonococcal (GC) medium base or chocolate agar supplemented with 1% Kellogg's defined supplement) prior to being used for additional confirmatory tests (38, 130).

From cultured bacteria, it is important to distinguish between *N. gonorrhoeae* and other commensal *Neisseria* species. Thus, the primary selection of cultured isolates is based on colony morphology, Gram stain and oxidase testing (125-127). For confirmatory testing, biochemical tests, chromogenic enzyme substrates and serological tests are also used. A detailed description of these tests is beyond the scope of this discussion (2, 130).

A significant advantage of culture is that minimum inhibitory concentration (MIC) testing of

various antibiotics to *N. gonorrhoeae* is only possible with culture at present. MIC testing of *N. gonorrhoeae* is performed on GC medium base with additional supplements (i.e. Kellogg's defined supplement). MICs are traditionally determined either with agar dilution or disk diffusion methods (25). However, many laboratories now choose to use E-tests due to their ease of use (i.e. less labour intensive and faster compared to the Agar dilution method). When testing MICs for new isolates, a panel of isolates with well-established MIC values should always be used in parallel for comparison (38). Regardless of the method used, results should be evaluated against the Clinical and Laboratory Standards Institute (CLSI) or European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints for each antibiotic (25, 131)

1.4.3 Nucleic Acid Amplification Tests

In contrast to traditional diagnostic methods for *N. gonorrhoeae*, nucleic acid amplification tests (NAATs) provide superior sensitivity and specificity and are now the first choice for diagnosis in most high-income settings (Table 1.1). NAATs are more appealing to clinicians and for laboratory personnel due to their ease of specimen handling (i.e. viability of the organism is not a concern), non-invasive specimen collection (i.e urine and self-collected vaginal swabs), rapidity, simultaneous detection of multiple STI pathogens (i.e. *N. gonorrhoeae* and *C. trachomatis*) and a non-requirement for highly skilled laboratory technicians such as with culturing (123, 126, 132-134).

There are also many in-house PCR based molecular tests for gonococcal diagnosis (135-138). These tests are widely used in low-income settings and have not been validated by regulatory agencies such as the United States Food and Drug Administration (FDA) and the European Conformity (CE; 2). Almost all the NAATs used in high-income settings have been validated by regulatory agencies for their performance with various clinical specimens (Table 1.2).

NAATs are more sensitive to *N. gonorrhoeae* diagnosis than cultures. FDA and CE-*in-vitro* diagnostic (CE-IVD) approved NAATs for *N. gonorrhoeae* are grouped into three categories: PCR, strand displacement amplification, and transcription-mediated amplification (Table 1.2).

Table 1.2 FDA and CE-IVD approved Nucleic acid amplification tests for the diagnosis of *N. gonorrhoeae*. Table adapted from Unemo et al., 2019. Gonorrhoea. *Nat Rev Dis Primers*. 5:79. The table is reproduced with permission from *Nat Rev Dis Primers*.

Test, instrument (manufacturer)	**Gonococcal target(s)**	**Specimen Type or Cultured isolate**	**Sensitivity (%)**		**Specificity (%)**	
			Symptomatic	**Asymptomatic**	**Symptomatic**	**Asymptomatic**
PCR						
Real-Time CT/NG[a], m2000[b] (Abbott Molecular)	*opa*	SCVS (F)	96.7-98	95.7	99.7-100	100
		CCVS (F)	96.8	95.7	99.9	99.4
		ECS (F)	87.1	91.3	99.7	100
		FVU (F)	76.9	NA	99.8	NA
		Urine (F)	93.8	87	99.7	99.6
		Urethral (M)	99.2	81.8	99.3	99.8
		Urine (M)	98.8	100	99.5	100
		Urine (M/F)	100	NA	100	NA
		Culture	99.5	NA	100	NA
Xpert CT/NG[a,c,d], GeneXpert (Cepheid)[b]	Two (NG2, NG4) highly conserved, noncontiguous unique chromosomal targets	VS (F)	100	100	99.8	99.9
		ECS (F)	100	100	100	100
		Urine (F)	100	91.7	100	99.9
		Urine (M)	97.8	100	100	99.9
		Culture	100	NA	100	NA
Cobas 4800 CT/NG[a], Cobas 4800[b] (Roche)	Direct repeat region 9 (DR9)	SCVS (F)	84.6	NA	99.6	NA
		FVU (F)	81.1	NA	100	NA
		Urine (M/F)	92.9	NA	100	NA
		Urogenital (F)	97.5	NA	100	NA
		Urogenital (M)	100	NA	100	NA

		Nongenital (F)	100	NA	100	NA
		Nongenital (M)	100	NA	99.8	NA
		Culture	100	NA	100	NA
BD MAX[e], BD Max System (Becton-Dickinson)	Chromosomal DNA	VS	96.3	94.1	99.8	99.9
		ECS	96.3	94.1	99.9	100
		Urine (F)	100	88.9	99.9	99.5
		Urine (M)	NA	80	NA	100
Strand displacement amplification						
Probe Tec ET[e], Viper XTR (Becton-Dickinson)	Pilin gene-inverting protein homologue	SCVS (F)	90.6-100	NA	100	NA
		ECS (F)	87.5	91.3	99.6	98.9
		FVU (F)	75.5	NA	100	NA
		Urine (F)	76.7	85.7	95.6	96.9
		Urine (M)	94.9	100	97	95.7
		Urine (M/F)	95.8	NA	100	NA
		SCRS (M)	77.1	NA	99.3	NA
		CCRS (M)	67.5	NA	100	NA
		PS (M)	85.7	NA	100	NA
		Rectal (M)	89.1	NA	99.8	NA
		Culture	100	NA	88.9	NA
Transcription mediated amplification						
Aptima Combo 2[a,d], Panther[b] (Hologic (earlier Gen-Probe)	16S rRNA	SCVS (F)	96.2-100	NA	98.4-100	NA
		CCVS (F)	93.8	95.7	99.3	99.7
		ECS (F)	90.6	90.9	99.4	99.7
		FVU (F)	88	NA	99.4	NA
		Urine (F)	84.4	82.6	99.6	99.4
		Urethral (M)	99.2	81.8	99.2	99.7

		Urine (M)	97.9	100	99.7	99.5
		Urine (M/F)	100	NA	100	NA
		SCRS (M)	84.3	NA	100	NA
		CCRS (M)	78.3	NA	99.8	NA
		PS (M)	100	NA	99.6	NA
		Rectal (M)	93.5	NA	97.7	NA
		Culture	100	NA	100	NA

CCRS, clinician-collected rectal swab; CCVS, clinician-collected vaginal swabs; ECS, endocervical swab; FVU, first void urine; PS, pharyngeal swabs; SCRS, self-collected rectal swab; SCVS, self-collected vaginal swabs; VS, vaginal swabs; F, female; M, male; NA, not applicable.

[a]Can also detect *Chlamydia trachomatis*

[b]Fully automated

[c]Cartridge based near-point-of-care test

[d]FDA approved for extragenital specimens such as rectal and pharyngeal

[e]Can also detect *C. trachomatis* and *Trichomonas vaginalis*

PCR based NAATs include RealTime CT/NG (Abbott Molecular), Xpert CT/NG (Cepheid), Cobas 4800 CT/NG (Roche), and BD MAX (Becton-Dickinson). Strand displacement and transcription-mediated NAATs include Probe Tec ET (Becton-Dickinson) and Aptima Combo 2 (Hologic), respectively. Although many NAATs for *N. gonorrhoeae* diagnosis use genital specimens (Table 1.2), two of the most widely used NAATs (Aptima Combo 2 and Xpert CT/NG) have recently received FDA approval for extragenital specimen testing (139). Nonetheless, a second confirmatory target or test will be useful for pharyngeal specimens, as many commensal *Neisseria* species residing in the pharynx could provide false-positive results (133, 140).

A significant disadvantage of all the approved commercial NAATs is the inability to characterize an isolate's AMR profile. However, there are NAATs in development that can predict the *N. gonorrhoeae* susceptibility to ciprofloxacin and one such test, Resistance Plus GC (SpeeDx), has recently received FDA approval and is currently being used in the United States and Europe (13, 134). Furthermore, and especially due to the lack of AMR testing by approved NAATs, remnant NAAT specimens (i.e. remnant Aptima urine specimens from Hologic Aptima Combo 2 Assay) have been used by a few researchers to identify evolving AMR patterns in gonococci (141-146). Nonetheless, such testing has been hindered by nucleic acid (i.e. DNA) degradation due to the Aptima buffer (143, 147).

With the rise of NAATs, there has also been an increase in the number of gonorrhea infections diagnosed. In 2017, the Canadian National Microbiology Laboratory reported that 70% of gonorrhea cases in Canada were identified using NAATs (24, 148-150). The rise in gonorrhea infections is not only attributed to higher sensitivities of NAATs, but also to the larger number of specimens diagnosed per day and varying sexual practices and more patients seeking treatment due to more acceptance (especially in high-income settings) of their sexual orientations (i.e. MSM and bisexual individuals; 143, 151).

1.4.4 Point-of-Care Tests

Due to the widespread use of NAATs, AMR testing of *N. gonorrhoeae* isolates has significantly declined in resource-rich countries (6, 152). AMR testing is often beyond the technical capabilities of resource-limited settings because this requires expensive reagents and highly skilled personnel.

Although several studies have utilized remnant NAAT specimens, the focus was largely on either bacterial load detection or only explored a limited number of markers related to ESC or ciprofloxacin resistance (7-9). However, due to the lack of AMR surveillance globally, the total burden of *N. gonorrhoeae* AMR remains incomplete (5). A recent study noted ESC resistance in 9.4% isolates while 18.8% isolates were multidrug-resistant (153). This study highlights the importance of continuous surveillance to prevent the spread of AMR among gonococci.

This calls for a near-patient test (i.e. point-of-care tests; POCTs) that can identify an isolate's resistance profile to an antibiotic at local health settings. Rapid (i.e. real-time) determination of AMR status will facilitate evidence-based treatment and will fill the knowledge gap in *N. gonorrhoeae* AMR spread. This is especially valuable in low-income settings where treatment is largely based on syndromic management and patients may not return for diagnosis results (154-156). Although any test that provides a rapid diagnosis during a single clinical visit could be identified as a POCT (Table 1.3), ideally such tests would be approved by regulatory agencies such as the FDA and CE-IVD, such that there is an assurance of the reliability of results. Furthermore, the WHO has established that an ideal POCT should meet the ASSURED criteria, which states the test should be affordable, sensitive, specific, user-friendly, robust and rapid, equipment-free and deliverable to the end-users (155, 157, 158).

POCTs for *N. gonorrhoeae* diagnosis include optical immunoassays and immunochromatographic tests that rely on antigen detection, as well as PCR based tests (134, 158-160), such as the Xpert CT/NG assay which has shown promising results from South Africa, Papua New Guinea and underprivileged regions in Australia (134, 161-163). Unfortunately, optical immunoassay and immunochromatographic POCTs have demonstrated sensitivities and specificities as low as 12.5% and 80% respectively (158, 159). As a result, these tests are not recommended for gonococcal diagnosis. However, modelling studies have shown that a rapid diagnostic test with a lower sensitivity can significantly increase the number of patients treated rather than waiting for a highly sensitive, yet slower test or a highly sensitive but expensive test requiring substantial electricity such as the Xpert CT/NG assay (164).

Table 1.3 Point-of-care tests approved and in development for *N. gonorrhoeae*. Table adapted from Unemo et al., 2019. Gonorrhoea. *Nat Rev Dis Primers*. 5:79. The table is reproduced with permission from *Nat Rev Dis Primers*.

Platform	GeneXpert CT/NG	Binx io CT/NG	ID NOW CT/NG[a]	Truenat CT/NG	Resistance Plus GC[b]
Manufacturer	Cepheid	Atlas Genetics	Abbott	Molbio	SpeeDx
Instrument; health-care setting	Table-top, not portable (used in mobile clinics); Level 2[c]	Table-top, portable; Level 1[c]	Table-top, portable; Level 1	Table-top, portable; Level 1-2	Table-top, not portable; Level 2
Amplification technology	PCR	NAAT, immunoassay and small molecule chemistry	Isothermal PCR	Real-time PCR	Real-time PCR
Specimen	Female and male urine, endocervical swab, patient-collected vaginal swab	Self-collected and clinician-collected vaginal swabs from symptomatic and asymptomatic females, and urine from males.	TBD	Female endocervical and vaginal swabs, male urethral swab, male and female urine	Male and female urine, rectal, cervical, vaginal, urethral, pharyngeal, and ocular swabs, ocular extracts
Procedure	~4 steps, sample prep automated	~4 steps, sample prep automated	~6 steps, raw sample added to the device	Multiple pipetting steps	~4 steps
Time to result	~90 minutes	30 minutes	15 minutes	~60 minutes	50 minutes
Reagent stability	3 years	Cartridges with reagents stable at 2-25ºC	>12 months	2 years at temperatures 2-30ºC	18-24 months

Energy requirements	Mains power required; solar power possible and can be powered by 12V DC or 120V AC	Mains power required	AC mains and DC from external AC/DC supplied plug pack	Rechargeable lithium-ion battery	Mains power required
Training	Less than 0.5 days	Less than 1 hour	Less than 0.5 days	Less than 0.5 days	Less than 0.5 days
Connectivity	Yes, computer required, remote calibration	Yes, via middleware	Yes, USB and ethernet outlets	Yes, wireless connectivity: Wi-Fi, Bluetooth, SMS	Yes, computer required
Regulatory Compliance	FDA, CE-IVD	CE-IVD, FDA approval pending	N/A	CE-IVD approval pending	CE-IVD, FDA approval pending

Tests listed in this table are selected based on the amount of information available and is not a comprehensive list of all the POCTs for *N. gonorrhoeae* in development. TBD – to be determined; NA – not available.

[a]Previously named Alere i CT/NG

[b]First licensed molecular test for detecting *N. gonorrhoeae* and its AMS to ciprofloxacin

[c]Level 1 – primary healthcare center; Level 2 – district hospital

Although the current POCT platforms for *N. gonorrhoeae* can provide results within 30-90min, their sensitivities and specificities are highly variable between platforms (157), and despite the results being available promptly, for many of these tests it could take up to days for the diagnosis to be delivered to the patient (165, 166). These POC tests include GeneXpert CT/NG (Cepheid), Binx io CT/NG (Atlas Genetics), ID NOW CT/NG (Abbott), Truenat CT/NG (Molbio), and Resistance Plus GC (SpeeDx; Table 1.3). Additionally, multiple in-house near-patient POCTs have been developed to predict ciprofloxacin susceptibility (141-145, 147, 158, 167-173). Although many of these tests have been assessed with clinical specimens (141-146), most in-house tests have not been assessed in larger populations.

1.5 COUNTERMEASURES TO ADDRESS AMR IN *N. GONORRHOEAE*

The WHO's 2016-2021 global health sector strategy for STIs has highlighted global targets to address the STI epidemic. To this end, the Pan-Canadian Sexually Transmitted and Blood-Borne Infections (STBBI) Framework for Action was approved in 2018 (170, 174, 175), and includes reducing the cases of STBBIs in Canada, providing improved access and support for testing, treatment and care. One of the important strategies outlined in this initiative was the effort to reduce discrimination and stigma associated with an STBBI diagnosis (16, 175). Thus, providing improved NAATs and POCTs alone will not reduce the number of infections; rather, a collective effort to reduce stigma, educate young adults on safer sexual practices, and provide accessible testing and affordable treatment, together with isolate tracking and development of novel therapeutics and vaccines will contribute to the effective control of rising gonorrhea infections.

The introduction of novel antibiotics to the market is a lengthy process and requires regulatory approval at several levels. However, multi-drug resistant *N. gonorrhoeae* isolates are emerging all over the world. Thus, immediate and alternate strategies are critical to controlling the rise of AMR in *N. gonorrhoeae*. One seemingly feasible approach is the re-introduction of older antibiotics, for which a given gonococcal isolate is still susceptible. Ciprofloxacin is one prime example of such an antibiotic. However, prior to treatment, the susceptibility status of *N. gonorrhoeae* isolates to ciprofloxacin must be established. The FDA approved Resistance Plus GC (SpeeDx) is licensed

for use in the United States and Europe (134). However, this test is not accessible to resource-constrained settings. Therefore, a user-friendly and rapid diagnostic test that can identify *N. gonorrhoeae* infections and determine isolates' susceptibility to an antibiotic, such as the RT-PCR based tests developed during my research to determine *N. gonorrhoeae* susceptibility to ciprofloxacin, would be more applicable in a multitude of settings. Additionally, if such a test (or at least a *N. gonorrhoeae* diagnosis test) can be made portable, it would be highly beneficial for the diagnosis and control of drug-resistant *N. gonorrhoeae* infections.

1.6 HYPOTHESIS AND OBJECTIVES

1.6.1 Hypothesis

Multidrug-resistant *N. gonorrhoeae* is a global public health threat and can compromise the control and management of gonorrhea. Resistance to the ESCs, the last resort treatment of gonorrhea, was reported from high-income global settings. To prevent the onset of an era of untreatable gonorrhea, it is a paramount health priority to understand and monitor the spread of AMR. Priorities of the WHO global action plan to control the spread and impact of AMR in *N. gonorrhoeae* indicated the need for strengthened AMR surveillance and research into molecular methods for monitoring and detecting AMR, especially to develop and update treatment guidelines rapidly.

I hypothesize that real-time molecular diagnostic platforms with specific primers will allow timely diagnosis of *N. gonorrhoeae* and information on its resistance to various antimicrobials used for treatment. Furthermore, molecular characterization of resistant determinants will provide a perspective of the spread of AMR and the emergence of resistance in gonococci in local communities (i.e. Saskatchewan).

1.6.2 Objectives

The specific objectives of my thesis are:

1) To develop a multiplex real-time PCR assay for simultaneous identification of *N. gonorrhoeae* and its ciprofloxacin susceptibility status.
2) To evaluate a commercial hydrogel platform for the diagnosis of *N. gonorrhoeae* by, using *N. gonorrhoeae* specific diagnostic primers, and then using multiplex primers capable of discriminating between ciprofloxacin susceptible and resistant isolates.
3) To use molecular techniques and predict trends of antimicrobial resistance in *N. gonorrhoeae* isolates from Saskatchewan.
4) To characterize a potential novel resistance-conferring mutation in an *N. gonorrhoeae* isolate leading to slow β-lactamase production.

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CHAPTER 2: EVALUATION OF A HYDROGEL BASED DIAGNOSTIC APPROACH FOR THE POINT-OF-CARE BASED DETECTION OF *NEISSERIA GONORRHOEAE*

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2.0 INTERFACE

At present, there are several in-house and commercial diagnostic tests for *N. gonorrhoeae*. However, none of these tests are portable and even require a DNA extraction step prior to diagnosis. Many high gonorrhea burden communities are in resource-constrained settings and could benefit from a portable point-of-care test requiring minimal training. The portable and commercial hydrogel system used in this research has worked well for the diagnosis of multiple pathogens directly from clinical specimens, without a DNA extraction step. In this research, we assess the feasibility of using the hydrogel platform for *N. gonorrhoeae* diagnosis. Importantly, the primers used in this research are unique to *N. gonorrhoeae* and have not reported cross-reaction with any other *Neisseria* species or non-*Neisseria* species tested. This chapter was previously published in *Antibiotics*, 7(3): 70, 2018, and is reprinted here with permission. This work was

presented at the International Union Against Sexually Transmitted Infections (IUSTI) World + European Congress held in Dublin, Ireland on June 27-30th, 2018.

Sumudu R Perera performed laboratory experiments, validation and formal analysis of data, design methodology and drafted the original manuscript

Ali Taheri designed and validated the primers

Nurul H Khan performed preliminary laboratory experiments and data analysis, designed methodology

Rajinder P Parti involved in preliminary protocol design and funding acquisition

Stephanie Stefura, Pauline Skiba, and **Jason P Acker** developed and supplied the hydrogels

Irene Martin supplied the non-gonococcal DNA

Anthony Kusalik designed the primers and reviewed the manuscript

Jo-Anne R Dillon conceptualized the research, provided supervision and project administration, reviewed and edited drafts of the manuscript and was responsible for the final draft of the manuscript, and held the research grant that supported this work

2.1 ABSTRACT

Eleven primer pairs were developed for the identification of *N. gonorrhoeae*. The sensitivity and specificity of these primers were evaluated by Real-Time (RT)-PCR melt curve analyses with DNA from 145 *N. gonorrhoeae* isolates and 40 other *Neisseria* or non-*Neisseria* species. Three primer pairs were further evaluated in a hydrogel-based RT-PCR detection platform using DNA extracted from 50 *N. gonorrhoeae* cultures. We have observed 100% sensitivity and specificity in the hydrogel assay confirming its potential as a point-of-care test (POCT) for *N. gonorrhoeae* diagnosis.

2.2 INTRODUCTION

N. gonorrhoeae, the second most prevalent bacterial sexually transmitted infection (STI) globally, causes 78 million new gonorrhea infections annually (1). Rates of antimicrobial-resistant (AMR) gonorrhea are rising worldwide (2). Left untreated, gonococcal infections can cause severe and potentially life-threatening disease and reproductive complications, especially in women, including infertility or involuntary death of the fetus (2, 3). With no immunization available, the only way to eliminate the infection is with effective antibiotic therapy (4, 5).

Early diagnosis of infection, preferably at the patient's first contact with the health care system, is vital for proper control of *N. gonorrhoeae* (6). Traditional diagnosis of gonorrhea largely relied on microscopy and or culture of the organism from urogenital specimens. Highly sensitive and specific nucleic acid amplification tests (NAATs) have replaced these tests in resource-rich settings (7-10). In resource-poor settings, either the appropriate laboratory facilities are not available at the primary health care level, or the costs associated with the diagnosis are unaffordable. In these instances, patients are treated empirically based on symptoms (i.e. syndromic management) (11, 12). Syndromic management often fails to identify asymptomatic infections and leaves a large population of people at risk for ongoing transmission and severe complications (13, 14). Therefore, it is of the utmost importance to design rapid, easy to perform tests that can provide early diagnosis of *N. gonorrhoeae* even in asymptomatic patients.

An important factor contributing to the rising prevalence of gonorrhea is treatment delay whereby a patient may wait for a positive diagnostic test result before treatment is initiated (15). Sometimes, despite the availability of laboratory facilities, the delays in reporting results severely hinder timely treatment (6). It has been shown by mathematical modelling that a rapid test with a sensitivity as low as 63% can drastically increase the number of patients treated, rather than waiting for a highly sensitive, yet slower test (16). Likewise, although culture is considered the gold standard for a positive diagnosis, the number of infected individuals diagnosed by a rapid test and in turn successfully treated can outnumber the number of patients who return for treatment following extensive diagnostic procedures such as culturing and more sensitive NAATs (17). Furthermore, viable organisms are required for culturing. Improper handling and transportation can reduce the

viability of *N. gonorrhoeae* leading to false-negative results. However, with NAATs, the viability of the organism is not a concern (18, 19).

The development of point-of-care tests (POCTs) for an STI such as gonorrhea is recognized as a vital approach to improve timely diagnosis (13, 14, 20-22). Although POCTs are commercially available for the diagnosis of *N. gonorrhoeae*, many of these tests do not fit the ASSURED criteria important for STI diagnosis tests, such as affordability, sensitivity, specificity, user-friendliness, robustness and rapidness, equipment-free, and deliverable to the end-users (13, 21). A reliable and rapid diagnostic test for *N. gonorrhoeae* would be a critical advancement in diagnostic capability.

The portable hydrogel-based RT-PCR method, developed by Aquila Diagnostic Systems Inc. Edmonton AB, Canada, is a rapid diagnostic platform utilizing Real-Time Polymerase Chain Reaction (RT-PCR) melt curve analysis to amplify nucleic acids directly in clinical specimens and report the presence of specific genetic targets (23). This system has successfully detected blood-borne infectious diseases such as malaria, BK virus, and herpes simplex virus (23-25). We developed and evaluated eleven diagnostic primer pairs for the identification of *N. gonorrhoeae.* We then evaluated three of these primers with the hydrogel-based detection method to assess the potential of this method for further development as a POCT.

2.3 MATERIALS AND METHODS

2.3.1 Bacterial Strains

To determine the sensitivity and specificity of primer pairs developed by us for the identification of *N. gonorrhoeae*, DNA from 145 *N. gonorrhoeae* isolates was obtained from the Dillon Culture Collection and represented isolates from diverse geographical areas (26-28) (Table 2.1: Panel 1). These isolates included the World Health Organization (WHO) *N. gonorrhoeae* reference isolates M, L, F, B, O, C, P, K, G and N (29). A random collection of 40 other *Neisseria* and non-*Neisseria* species were obtained from the National Microbiology Laboratory (NML), Winnipeg MB, Canada (Table 2.1: Panel 1).

For experiments evaluating the hydrogel platform, DNA from 50 cultured *N. gonorrhoeae* isolates was used (Table 2.1: Panel 2 is a sub-panel of Panel 1). WHO *N. gonorrhoeae* reference strains WHO-F, P, G, K and N were used as positive controls. Five non-*N. gonorrhoeae* and non-*Neisseria* species (*E. coli*, *L. jensenii*, *N. elongata*, *N. subflava*, and *S. enterica* serovar Typhimurium) were used as negative controls.

2.3.2 DNA Extraction

The DNA extraction was performed with a QIAamp DNA Mini Kit (#51306 Qiagen Canada) according to the manufacturer's instructions. DNA was quantified using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington DE, USA) and the final concentration was adjusted to 50, 70 or 100ng/µL for evaluating the hydrogel method.

2.3.3 Design of Diagnostic Primer Pairs

Non-homologous sequence regions between *N. gonorrhoeae* FA1090 and *N. meningitidis* (MC58, FAM18 and Z2491) were extracted from the *N. gonorrhoeae* FA1090 genome. These sequences were then BLAST-ed against 25 in-house sequenced *N. gonorrhoeae* genomes and 15 *N. gonorrhoeae* genomes available at the Broad Institute database. Sequences that were positive in this BLAST search (50 sequences) were then further aligned against the NCBI non-redundant (nr) database using the BLAST program. Regions that were only found in *N. gonorrhoeae* were filtered for further analysis. Targets for all primer pairs are present in multiple copies in the *N. gonorrhoeae*

FA1090 genome and in-turn increase the sensitivity of detection even at lower DNA concentrations. Based on these findings, eleven primer pairs were designed using Primer-BLAST (36) (Table 2.2). Potential cross-reactivity of the primer pairs was evaluated *in silico* using primer-BLAST software against the NCBI-nr database. Primer pairs were tested *in vitro* for their sensitivity and specificity with *N. gonorrhoeae*, non-*N. gonorrhoeae* and non-*Neisseria* species.

2.3.4 Limit of Detection of Primers

RT-PCR reactions contained 5 µl of 2X SYBR-Green master mix (Cat # 4472912, Life Technologies Inc.), 0.25 µl of each primer (10µM), 1 µl of DNA (50ng/µl) and 3.5 µl PCR-grade water in a total 10 µl reaction volume. Initial holding and activation of DNA polymerase were at 50°C for 2 minutes. RT-PCR was performed for 40 cycles of 95°C for 20 seconds and 60°C for 40 seconds. A post PCR melt curve was performed between 60°C to 95°C with 0.3°C temperature increments. Limit of detection assays, using serial dilutions (100 ng/µl to 0.00001 ng/µl) of DNA from *N. gonorrhoeae* reference strains WHO F, P, G, K and N, were used to ascertain the sensitivity of each primer pair. DNA from 40 non-*N. gonorrhoeae* and non-*Neisseria* species was also used to test the specificity of each primer pair.

2.3.5 Real-Time PCR and Hydrogel Methods

To compare hydrogel and RT-PCR methods, DNA was amplified using three *N. gonorrhoeae* diagnostic primer pairs (3, 17-1, and 21-5) and SYBR Select master mix. For the RT-PCR control method, each reaction contained 2 µL DNA (50, 70 or 100 ng/µL), 1.5 µL of each primer (10 µM) and 5 µl 2X SYBR-Green master mix in a total 10 µl reaction volume. For the hydrogel method, each reaction contained 1 µl DNA (50, 70 or 100 ng/µL), 0.38 µL of each primer (10 µM) and 8.24 µL PCR-grade water. 2X SYBR-Green and primers were pre-mixed with the hydrogel. DNA and water were added to the hydrogel immediately before the start of the RT-PCR assay. Initial holding and activation of DNA polymerase were at 50°C for 2 minutes. RT-PCR was then performed for 30 cycles of 95°C for 20 seconds and 60°C for 40 seconds. A post PCR melt curve was performed between 60°C to 95°C with 0.3°C temperature increments. Various RT-PCR conditions were tested to optimize the hydrogel method. The final assay conditions included: 30 PCR cycles, 60^{o}C annealing temperature, 60-95^{o}C melt curve temperature range, 0.3^{o}C image taking temperature increments, and 2x SYBR-Green concentration.

Table 2.1 *N. gonorrhoeae*, non-*N. gonorrhoeae* and non-*Neisseria* isolates used in this research.

Isolate selection	Organisms	Geographic source	No	References
Panel 1	*N. gonorrhoeae*	Saskatchewan	86	26, 27
		USA	13	Dillon Culture Collection
		China	8	28
		WHO	10	29
		South America and the Caribbean	28	Dillon Culture Collection
	Non-*N. gonorrhoeae*[b]	Canada	30	NML[a]
	Non-*Neisseria* species[b]	Canada	10	NML
Panel 2	*N. gonorrhoeae*	Saskatchewan	35	26, 27
		China	6	28
		South America and the Caribbean	9	Dillon Culture Collection
		WHO	5	29
	Non-*N. gonorrhoeae*[c]	Canada	2	NML
	Non-*Neisseria* species[c]	Canada	3	NML

[a]National Microbiology Laboratory
[b]One isolate of – *Enterococcus faecalis*, *Enterococcus faecium*, *Escherichia coli*, *Klebsiella oxytoca*, *Lactobacillus jensenii*, *Moxarella catarrhalis*, *Neisseria animaloris*, *Neisseria cinerea*, *Neisseria elongata*, *Neisseria flava*, *Neisseria lactamica*, *Neisseria menengitidis*, *Neisseria mucosa*, *Neisseria perflava*, *Neisseria polysacchareae*, *Neisseria sicca*, *Neisseria subflava*, *Neisseria wadsworthii*, *Neisseria weaverii*, *Pseudomonas aeruginosa*, *Salmonella enterica* serovar Typhimurium, *Staphylococcus aureus*, and *Staphylococcus epidermis*
[c]One isolate of – *E. coli*, *L. jensenii*, *N. elongata*, *N. subflava*, and *S. enterica* serovar Typhimurium

Table 2.2 Primer sequences used in this research for detection of *N. gonorrhoeae*, their respective targets and copy numbers present in the *N. gonorrhoeae genome*.

Primer ID (product length)	Sequence (5'->3')	Target Gene[b]	No Targets in FA1090[c]	Similar Targets from Commercial NAATs
Primer 2 (208bp)		NGO1620, NGO0469, NGO1126	3	Cobas 4800 CT/NG
Forward	TCTGCTTTCTTGGTGGGCGA			
Reverse	AGGCGATCCGGAAATGCTGA			
Primer 3 (139bp)		NGO05940, NGO06090, NGO06650, NGO1642	4	-
Forward	TATGGGGGTTCCTTCGCACC			
Reverse	CAGACGGTTGCGGGTTCTTG			
Primer 8-3 (132bp)		NGO0773, NGO1200, NGO1703, NGO1137, NGO1164, NGO1262, NGO1641	7	BD ProbeTec GC Qx
Forward	CAGAAGCCTACGGACGAGCA			
Reverse	CGCATATGCTTTGGCCGCTT			
Primer 8-4 (180bp)		NGO0773, NGO1200, NGO1703, NGO1137, NGO1164, NGO1262, NGO1641	7	-
Forward	TCACGGATGACCGCAGCATA			
Reverse	AGACGCTTCACGCCTTCCTT			
Primer13 (138bp)		NGO0773, NGO1137, NGO1703, NGO1164, NGO1200, NGO1262, NGO1641	7	BD ProbeTec GC Qx
Forward	GCGTAACGCCGTAGGATTGGA			
Reverse	CCCAAGCTTTTCAACCGGTCC			
Primer16 (93bp)		NGO1131, NGO1209	2	-
Forward	CGGAACAAGCGTTTTTCAGCG			
Reverse	TCTTTGGCTTGTCCGGGTGT			

Primer 17-1 (73bp)		NGO1638, NGO0487, NGO1108	3	-
Forward	TCCGAAACACGCAAACCGAAA			
Reverse	TAGCCCGGGTTGGTATTGCC			
Primer 17-2 (82bp)		NGO1638, NGO0487, NGO1108	3	-
Forward	ACACGCAAACCGAAACCGTC			
Reverse	GCGCGGTTTTTGTAATAGCCC			
Primer 21-5 (101bp)		NGO1085, NGO1652	2	-
Forward	GCACGAAACCCGTCCAATCC			
Reverse	CAAGACATGCGGCTATGCGG			
Primer 31-2 (188bp)		NGO0480, NGO1113, NGO1631	3	-
Forward	AAAATCGCGCCGGGTTTGAA			
Reverse	AGCTTATCCGCAGCGGTTCT			
Primer 31-3 (275bp)		NGO0480, NGO1113, NGO1631	3	-
Forward	AAAAAGCCCGTCGGGTCAGA			
Reverse	AACCCGAAGAATCGGAGCCA			

[a]The primer sequences presented in this manuscript are the subject of a United States utility patent (#62/088,332)
[b]Locus Tag ID in the NCBI database
[c]Number of targets on *N. gonorrhoeae* FA1090 genome

2.4 RESULTS

2.4.1 Evaluation of *N. gonorrhoeae* Diagnostic Primer Pairs

Nine primer pairs (3, 8-3, 8-4, 13, 16, 17-1, 17-2, 21-5, 31-2) amplified every *N. gonorrhoeae* isolate tested (n=130) (Table 2.3). Primer pair 2 did not amplify one of the 130 *N. gonorrhoeae* isolates. Primer pair 31-3 did not amplify 28 of the 130 *N. gonorrhoeae* isolates. Primer pairs 2, 3, 8-4, 16, 17-1, 17-2, 21-5, 31-2, and 31-3 did not amplify any of the non-*N. gonorrhoeae* or non-*Neisseria* species. However, primer pair 8-3 amplified DNA from *Neisseria flava*, *Neisseria lactamica*, and *Neisseria polysacchareae*. Primer pair 13 amplified DNA from *N. flava*, *Neisseria subflava*, *Neisseria mucosa*, *N. lactamica*, *Neisseria perflava*, *Neisseria flavescens* and *N. polysacchareae* (Table 2.4). The limit of detection of the primers was assessed by serially diluting (10 ng/µl to 0.000001 ng/µl) genomic DNA from *N. gonorrhoeae* strain WHO-M. After 30 cycles of PCR, all the primers were able to detect gonococcal concentrations as low as 0.001 ng/µl (data not shown).

2.4.2 Optimization of the Hydrogel System and Comparison with RT-PCR Methods

The ability of primers 3, 17-1 and 21-5 to identify genomic DNA from *N. gonorrhoeae* strains WHO F, WHO P, WHO G, WHO K and WHO N in the hydrogel system were analyzed using RT-PCR (Table 2.4). To optimize the performance of the hydrogel system for *N. gonorrhoeae*, different parameters for hydrogel and RT-PCR were assessed. In testing for the most effective concentration of SYBR-Green, we determined that with 1X SYBR-Green (pre-mixed in the desiccated gel), primer pair 3 produced melt curve temperatures (Tm values) similar to previously established Tm values (80.5^{o}C) with control methods. However, with primer pairs 17-1 and 21-5, 2x SYBR-Green was required for established Tm values (85.0^{o}C). Therefore, 2x SYBR-Green was chosen for subsequent experiments. Further, with our primer pairs, we determined that optimal amplification occurred with 30 PCR cycles, which differs from the hydrogel manufacturer's recommendation of 40 cycles.

The performance of the hydrogel method with increasing concentration of DNA (50, 70 or 100ng/µL) was assessed using genomic DNA from 50 *N. gonorrhoeae* positive cultures (Table 2.1: Panel 2), WHO *N. gonorrhoeae* reference strains (n=5; positive control) and

Table 2.3 Evaluation of eleven primer pairs using *N. gonorrhoeae,* non-*N. gonorrhoeae* and non-*Neisseria* species.

Bacterial species	**No. of isolates**	**Positive Amplifications**										
		2[a]	**3**	**8-3[b]**	**8-4**	**13[b]**	**16**	**17-1**	**17-2**	**21-5**	**31-2**	**31-3**
N. gonorrhoeae	130	129	130	130	130	130	130	130	130	130	130	102
N. flava	2	0	0	**1**	0	**2**	0	0	0	0	0	0
N. subflava	2	0	0	0	0	**1**	0	0	0	0	0	0
N. elongata	2	0	0	0	0	0	0	0	0	0	0	0
N. mucosa	3	0	0	0	0	**1**	0	0	0	0	0	0
N. lactamica	4	0	0	**2**	0	**2**	0	0	0	0	0	0
N. perflava/sicca	5	0	0	0	0	**3**	0	0	0	0	0	0
N. flavescens[c]	2	0	0	0	0	**1**	0	0	0	0	0	0
N. polysacchareae	4	0	0	**1**	0	**2**	0	0	0	0	0	0
Other *Neisseria* species[d]	6	0	0	0	0	0	0	0	0	0	0	0
Non-*Neisseria* species[e]	10	0	0	0	0	0	0	0	0	0	0	0

[a]Primers are amplifying regions with partial homology to DR9 repeats used in Cobas 4800 CT/NG Test (Roche Molecular Diagnostics, Pleasanton CA, USA)

[b]Primers are amplifying regions with partial homology to targets used in BD ProbeTec GC Q[x] Amplified DNA Assay (Becton Dickinson and Company, Franklin Lakes NJ, USA)

[c]*Neisseria flavescens*

[d]*Neisseria animaloris* (1), *Neisseria cinerea* (2), *Neisseria menengitidis* (1), *Neisseria wadsworthii* (1), *Neisseriaweaverii* (1)

[e]*Enterococcus faecalis* (1), *Enterococcus faecium* (1), *Escherichia coli* (1), *lebsiella oxytoca* (1), *Lactobacillus jensenii* (1), *Moraxella catarrhalis* (1), *Pseudomonas aeruginosa* (1), *Salmonella enterica* serovar Typhimurium (1), *Staphylococcus aureus* (1), and *Staphylococcus epidermis* (1)

Table 2.4 The effect of SYBR-Green concentration on melt curve temperature (Tm values) of five *N. gonorrhoeae* strains, with primer pairs 3, 17-1 and 21-5.

Strain	Melt Curve Temperature (ºC)					
	Primer Pair 3[a]		Primer Pair 17-1[a]		Primer Pair 21-5[a]	
	1X SYBR-Green	2X SYBR-Green	1X SYBR-Green	2X SYBR-Green	1X SYBR-Green	2X SYBR-Green
WHO-F	80.49	81.52	83.90	84.64	84.05	84.84
WHO-G	80.78	81.82	84.05	84.64	83.90	84.79
WHO-K	80.49	81.97	84.05	84.49	84.20	84.79
WHO-N	80.63	81.82	83.90	84.49	84.05	84.79
WHO-P	80.93	81.97	83.90	84.64	84.05	84.79

[a]Expected Tm values for primer pair 3 - 80.5ºC; primer pair 17-1 - 85.0ºC; primer pair 21-5 - 85.0ºC

non-*N. gonorrhoeae* strains (n=4; negative control). Based on the melt curve (Tm) temperatures (primer pair 3, 80.5^{o}C; primer pairs 17-1 and 21-5, 85.0^{o}C), primer pair 3 with 50 ng/µL DNA identified 44 *N. gonorrhoeae* isolates. Primer pair 3 with 70 ng/µL DNA identified 46 isolates and 100 ng/µL DNA identified all 50 *N. gonorrhoeae* isolates (Table 2.5). Thus, primer pair 3 had 88%, 92% and 100% sensitivity with 50, 70 and 100 ng/µL DNA respectively. The specificity of primer pair 3 was 100% with all DNA concentrations tested. Primer pair 17-1, irrespective of the DNA concentration tested, identified all 50 *N. gonorrhoeae* isolates and had 100% sensitivity and specificity. Primer pair 21-5 identified 47 *N. gonorrhoeae* isolates with 50 ng/µL DNA and had a sensitivity of 94% and a specificity of 100%. With 70 ng/µL and 100 ng/µL DNA, primer pair 21-5 identify all 50 isolates and had 100% sensitivity and specificity. Primer pairs 3 and 17-1 in the control RT-PCR method, identified all 50 *N. gonorrhoeae* isolates and had a sensitivity of 100%. Primer pair 21-5 identified 48 isolates with a sensitivity of 96%. One non-*Neisseria* species (*Escherichia coli*) was incorrectly identified as *N. gonorrhoeae* positive by primer pair 3 in the RT-PCR control method, thereby giving a specificity of 67% (Table 2.5). With primer pairs 17-1 and 21-5, all the negative control isolates (*E. coli, Lactobacillus jensenii, Neisseria elongata, Neisseria subflava,* and *Salmonella enterica* serovar Typhimurium) were correctly ascertained as *N. gonorrhoeae* negative, giving a specificity of 100%.

With the hydrogel method and primer pair 3, 33 samples had positive threshold cycle (Ct) values when using 70 ng/µL DNA. However, with 50 ng/µL and 100 ng/µL DNA, none of the samples had positive Ct values. Similarly, with primer pairs 17-1 and 21-5 all 50 samples had negative Ct values. In contrast, when using the control RT-PCR method and primer pair 3, all 50 samples had positive Ct values. Likewise, with primer pair 17-1 all 50 samples had positive Ct values. With primer pair 21-5, 49 samples had positive Ct values. To consider if the hydrogel system was interfering with the fluorescence reading, extra, fresh SYBR-Green (commercially made 2X SYBR-Green) was added to the reaction mixture of samples with the hydrogel method. These reaction mixtures pre-contained 2X SYBR-Green incorporated into the desiccated gel. The addition of total 4X and fresh SYBR-Green significantly improved the results from the hydrogel method and produced Ct values similar to that obtained with the RT-PCR control method (Table 2.5).

Table 2.5 Evaluation of hydrogel and RT-PCR methods using varying concentrations of *N. gonorrhoeae* DNA (n=50)[a] and diagnostic primer pairs 3, 17-1 and 21-5.

Primer Pair	Method	DNA Conc. (ng//µL)	No *N. gonorrhoeae* Isolates Identified as per Tm value (n=50)[b]	Positive Ct values (n=50)[b]	Sensitivity (%)	Specificity (%)
3	Hydrogel	50	44	0	88	100
3	Hydrogel	70	46	33	92	100
3	Hydrogel	100	50	0	100	100
3	Hydrogel+4X SYBR[d]	100	50	**50**	100	100
3	RT-PCR control	70	50	50	100	67[c]
17-1	Hydrogel	50	50	0	100	100
17-1	Hydrogel	70	50	0	100	100
17-1	Hydrogel	100	50	0	100	100
17-1	RT-PCR control	70	50	50	100	100
21-5	Hydrogel	50	47	0	94	100
21-5	Hydrogel	70	50	0	100	100
21-5	Hydrogel	100	50	0	100	100
21-5	RT-PCR control	70	48	49	96	100

[a]Controls - WHO *N. gonorrhoeae* reference strains (n=5; positive control) and non-*N. gonorrhoeae* and non-*Neisseria* strains (n=4; negative control)

[b]Number of *N. gonorrhoeae* isolates identified as per the melt curve temperature (Tm) and threshold cycle (Ct) values. Isolates with negative Ct values had a positive melt curve temperature (Tm)

[c]One Non-*N. gonorrhoeae* isolate identified as *N. gonorrhoeae* positive

[d]Hydrogel method pre-contained 2X SYBR-Green dye. For this assay, extra, fresh 2X SYBR-Green dye was added for a final concentration of 4X SYBR-Green dye

2.5 DISCUSSION

We developed and evaluated novel primers in a hydrogel platform as a potential useful POCT for *N. gonorrhoeae* diagnosis. This system is rapid and requires minimal sample preparation. The hydrogel system introduces a state-of-the-art portable diagnostic approach to bringing the detection platform to clinicians and remote locations, as this system contains desiccated primers, polymerases and other PCR components, thereby enabling the delivery of prompt results to clinicians. Only the patient sample would need to be added to the system prior to PCR amplification. As such, this system does not require a cold chain (i.e. refrigeration) to preserve the PCR reagents, a major obstacle to delivering diagnostic tests to remote locations.

A recent review evaluated POCTs for *N.* gonorrhoeae (13). The tests evaluated include lateral flow immunochromatographic test (ICT) formats, i.e. the Binax NOW Gonorrhoea Test (Inverness), the GC Check (PATH, Seattle, Washington, USA) and, the ACON format tests (ACON Laboratories, San Diego, California, USA); an optical immunoassay test (OIA), the BioStar (ThermoFisher/BioStar, Boulder, Colorado, USA); and a nucleic acid amplification test, the GeneXpert CT/NG Assay (Cepheid, Sunnyvale, California, USA). The performance of the ICTs was variable with sensitivities ranging from 94%, 70% and 12.5% and specificities from 96%, 97%, and 99.8% for Binax NOW, GC Check and ACON format tests, respectively. The OIA BioStar test had a sensitivity of 60% and a specificity of 89%. The ICTs and the OIA had 5 or more steps and took 25 to 40 minutes to obtain results (13, 14). These tests generally fail to meet the high levels of sensitivity and specificity required for a POCT. The GeneXpert CT/NG Assay is the only commercial NAAT-based POCT for *N. gonorrhoeae* diagnosis and has high sensitivity (95.6-98%) and specificity (99.9-100%) which is dependent on the specimen type (13, 30-33). This assay requires 3 steps and takes 90 minutes to produce results (31). The limiting factor about considering the GeneXpert as a POCT is the affordability and portability of the equipment.

The currently available, United States Food and Drug Administration approved commercial NAAT systems for *N. gonorrhoeae* identification primarily rely on a single target detection strategy (34). These tests include the Abbott RealTime CT/NG assay which targets a 48bp sequence within the *N. gonorrhoeae opa* gene (Abbott Laboratories Abbott Park, IL, USA), the

Aptima COMBO 2 assay and the Aptima GC assay which target specific regions within *N. gonorrhoeae* 16S rRNA (Hologic/Gene-Probe Inc., San Diego, CA, USA), the BD ProbeTec ET CT/GC Amplified DNA assay and BD ProbeTec Qx GC Amplified DNA Assay which target the chromosomal pilin gene-inverting protein homologue (Becton Dickinson and Company, Sparks, MD, USA), the Cobas 4800 CT/NG which targets the DR-9 region (Roche Diagnostics, Indianapolis, IN, USA) and the GeneXpert CT/NG Assay (Cepheid, Sunnyvale, CA, USA) which uses two non-contiguous chromosomal targets (NG2 and NG4) of *N.* gonorrhoeae (7, 30, 31, 34, 35).

The hydrogel method we evaluated had 100% sensitivity and specificity using DNA from *N. gonorrhoeae* cultures. The system remains to be tested using clinical specimens of *N. gonorrhoeae* such as urines or self-collected vaginal or rectal swabs. One complication that arose with the hydrogel method was that, despite having a positive *N. gonorrhoeae* diagnosis by melt curve (Tm) analysis, most samples contained negative threshold cycle (Ct) values. Therefore, we considered that the hydrogel system was interfering with the fluorescence reading, thereby giving Ct values lower than the threshold value (i.e. negative Ct values). Addition of extra, fresh 2x SYBR-Green to the hydrogel reaction mixture, pre-containing 2X SYBR-Green, for a final concentration of 4X SYBR-Green resulted in favourable Ct values. The hydrogel has been successfully used for the detection of other pathogens such as Herpes simplex virus from raw genital swabs, *Plasmodium falciparum*, BK virus, human platelet antigen 1 (HPA_1) and fibroblast growth factor receptor 2 ($FGFR_2$) from unprocessed blood samples (23-25). Furthermore, the <2-hour time frame from specimen collection to results makes this test a potentially much simpler and user-friendly second molecular POCT.

The genomic targets of three of our primer pairs have partial homology with genomic targets used in two commercial assays. Primer pair 2 has partial homology with the genomic region used in the Cobas 4800 CT/NG Test (i.e DR-9 region) and primer pairs 8-3 and 13 have partial homology with the genomic targets used in the BD ProbeTec GC Q^X Amplified DNA Assays (i.e. chromosomal pilin gene-inverting protein homologue). In our assay, primer pair 2 did not amplify one *N. gonorrhoeae* isolate. Furthermore, cross-reactivity of primer pairs 8-3 and 13 with 3 and 7 other non-gonococcal *Neisseria* species respectively was observed. Cross-reactivity with *Neisseria*

subflava and *Neisseria lactamica* was also reported for the BD ProbeTec GC Q^x Amplified DNA Assay (8).

2.6 CONCLUSIONS

The hydrogel method coupled with the primers discussed in this work can meet the ideal rapid test (ASSURED) criteria established by WHO for STI diagnosis tests; Affordable, Sensitive, Specific, User-friendly, Robust and Rapid, Equipment-free, and Deliverable to end-users (21). Although the hydrogel system was evaluated using pure cultures of *N. gonorrhoeae*, with further optimization the hydrogel diagnostic method with these primers can be used as a POCT for diagnosis of gonococci from clinical specimens enabling rapid, reliable and efficient diagnosis of *N. gonorrhoeae*. Furthermore, multiplex primer pairs can be integrated into the hydrogel platform enabling simultaneous diagnosis of *N. gonorrhoeae* and characterization of its AMR profile to different antimicrobials.

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2.9 CONFLICTS OF INTEREST

SS, PS and JPA are employees of and hold shares in, Aquila Diagnostic Systems Inc. The authors declare no other conflicts of interest.

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CHAPTER 3: A MULTIPLEX RT-PCR ASSAY FOR THE SIMULTANEOUS IDENTIFICATION OF *NEISSERIA GONORRHOEAE* AND ITS CIPROFLOXACIN SUSCEPTIBILITY STATUS

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3.0 INTERFACE

In North America, ciprofloxacin is not recommended for the treatment of *N. gonorrhoeae* infections due to a high prevalence of antimicrobial resistance. However, previous research has indicated that ciprofloxacin susceptible *N. gonorrhoeae* isolates exist in some communities. Therefore, we designed multiplex primers specific for *N. gonorrhoeae*, which can also identify a gonococcal isolate's ciprofloxacin susceptibility status, with the long term intention of introducing this test as a diagnostic tool to local health communities. This research highlights that ciprofloxacin susceptible *N. gonorrhoeae* isolates can be identified for targeted treatment, and is unique in that it emphasizes the importance of looking at mutations in both *gyrA* S91 and D95 amino acid

positions to determine resistance to ciprofloxacin. This chapter was previously published in the *Journal of Clinical Microbiology*, 55(11): 3201-3209, 2017, and is reprinted here with permission. The work discussed in this chapter has been presented at the 17th World Congress of the International Union against Sexually Transmitted Infections held in Marrakech, Morocco, on May 9-12th, 2016, and at the Saskatchewan Epidemiology Association 19th Annual Fall Symposium and Workshop held in Regina, Saskatchewan, Canada, on October 8-9th, 2019.

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Nurul H Khan carried out laboratory experiments and analyzed data
Irene Martin provided non-gonococcal DNA and performed validation experiments
Ali Taheri and **Anthony Kusalik** designed the primers
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Paul N Levett and **Greg B Horsman** provided gonococcal isolates
Jo-Anne R Dillon conceptualized the research, provided guidance with the experimental design, feedback and editing on the manuscript and held the research grant that supported this work

3.1 Abstract

A real-time PCR (RT-PCR) assay was designed for the simultaneous identification of *N. gonorrhoeae* and its ciprofloxacin susceptibility status. A SYBR-Green based multiplex RT-PCR format was used and comprised of two different forward primers and a common reverse primer to detect single nucleotide polymorphisms (SNPs) in *gyrA* of *N. gonorrhoeae.* The primer pairs were evaluated for their sensitivity and specificity with genomic DNA from 254 *N. gonorrhoeae* isolates (82 ciprofloxacin susceptible and 172 resistant) and 23 non-*N. gonorrhoeae* species. The performance of the primers was validated using genomic DNA from 100 different *N. gonorrhoeae* isolates (46 ciprofloxacin susceptible and 54 resistant) and 52 non-*N. gonorrhoeae* isolates. This latter panel was revalidated by testing 99 of the *N. gonorrhoeae* isolates (46 ciprofloxacin susceptible and 53 resistant) and 23 non-*N. gonorrhoeae* isolates. These primers detected *N. gonorrhoeae* and its ciprofloxacin susceptibility status with over 99% sensitivity and specificity for all panels tested. This assay has the potential to be an inexpensive and rapid test for the simultaneous identification of *N. gonorrhoeae* and its ciprofloxacin susceptibility status.

3.2 INTRODUCTION

Gonorrhea remains an important global public health concern with 78 million new infections worldwide each year (1). Cases of gonorrhea are underreported especially because most infections (almost half of the women infected) are asymptomatic and such infections can have major long-term and negative impacts on reproductive health, especially in women (2). Gonococcal infections can only be cured with antibiotics; there are no vaccines against this disease (3, 4). Over time, *N. gonorrhoeae* isolates have developed resistance to every class of antibiotic introduced for therapy, including the third-generation cephalosporin which was the last class of antibiotic used in single-dose therapy (3, 5). The recommended treatment in many countries for gonococcal infections now comprises co-therapy with two antibiotics, azithromycin coupled with ceftriaxone (6). There is an increased urgency for new strategies to treat gonococcal infections, either with new antibiotics, antibiotic combinations, or possibly to use older antimicrobials where susceptibility to the agent is known.

The identification of *N. gonorrhoeae* in high-income countries is usually accomplished by nucleic acid amplification testing (NAAT; 7, 8, 9, 10). A limitation of NAATs is that susceptibility testing cannot be completed at present as such testing requires the pathogen to be cultured, and molecular testing for antimicrobial susceptibility is still under evaluation (3, 5, 11, 12). Due to the widespread use of NAATs, antimicrobial susceptibility testing of *N. gonorrhoeae* isolates has significantly declined in resource-rich countries (12, 13). Such testing is often beyond the technical capability of resource-limited settings.

Ciprofloxacin, a fluoroquinolone antibiotic, was first introduced for the treatment of gonococcal infections in 1985; by 2006, in the United States, ciprofloxacin resistance was observed in 13.8% of isolates (11, 14). The population-based recommendation of the World Health Organization is that when resistance to an antibiotic exceeds 5% of isolates tested, the antibiotic should be discontinued for treatment (5, 6). Consequently, from 2007 ciprofloxacin has not been recommended for the treatment of *N. gonorrhoeae* infections in the USA (15). In Canada, between 2004 and 2014, ciprofloxacin resistance in *N. gonorrhoeae* isolates increased from 6.3% to 34.0% and is no longer recommended for the primary treatment of gonorrhea (16, 17). It is notable,

however, that in the United States, ~80% of *N. gonorrhoeae* isolates are susceptible to ciprofloxacin (18). The issue is that individual patients infected with a susceptible isolate must be identified before the antibiotic can be considered as a treatment option. Thus, there is a need for a point-of-care test (POCT) that might identify those infected with ciprofloxacin susceptible *N. gonorrhoeae* isolates.

Ciprofloxacin resistance in *N. gonorrhoeae* is caused by single nucleotide polymorphisms (SNPs) within DNA gyrase A (*gyrA*; amino acid positions S91 and D95) and *parC* (amino acid positions S88 and E91) (3, 5, 19, 20). Studies have shown that more than 99% of ciprofloxacin-resistant *N. gonorrhoeae* isolates carry *gyrA* S91 and/or D95 mutations, making these targets potentially diagnostic for resistance (21, 22, 23).

We report on the design and evaluation of a test that can be used simultaneously to identify *N. gonorrhoeae* and to determine its ciprofloxacin susceptibility status in a single multiplex assay. Our test was over 99% sensitive and specific for the identification of ciprofloxacin susceptibility status of *N. gonorrhoeae*.

3.3 MATERIALS AND METHODS

3.3.1 Bacterial Strains

Two different *N. gonorrhoeae* strain panels were used and comprised of DNA extracted from clinically derived cultured specimens. Panel 1 comprised 254 gonococcal and 23 non-gonococcal isolates (Table 3.1). Panel 1 *N. gonorrhoeae* isolates were chosen randomly from the Dillon culture collection, which consisted of isolates previously reported by Vidovic et al., 2014 (35) and Liao et al., 2009 (36). A core feature of all selections was their different susceptibilities to ciprofloxacin. The WHO reference isolates were reported in Unemo et al., 2009 (24). A random collection of non-*Neisseria* and other *Neisseria* species were obtained from the National Microbiology Laboratory (NML), Winnipeg MB, Canada. The second unrelated panel comprised 100 gonococcal and 52 non-gonococcal isolates from across Canada and was selected at the NML to include isolates having different susceptibilities to ciprofloxacin. A subsample of panel 2 (panel 3) containing DNA from 99 gonococcal and 23 non-gonococcal isolates was used for re-validation. One isolate from panel 2 was eliminated in panel 3 due to inconsistent results. WHO *N. gonorrhoeae* reference strains for ciprofloxacin susceptibility determination included strains F, O, P, B and C (susceptible), G (intermediate) and M, N, K and L (resistant; 24).

N. gonorrhoeae isolates were stored at -80°C in brain heart infusion medium (Difco BD Bioscience) with 20% glycerol. Aliquots from frozen inoculum were cultured on GC medium base (Difco BD Bioscience) supplemented with 1% Kellogg's defined supplement. Plates were incubated at 35-37°C with 5% CO_2 in a humid environment for 18 to 24h (25).

DNA extraction was performed using a QIAamp DNA Mini kit (Qiagen) according to the manufacturer's protocol. DNA was quantified using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific) and 50ng/µl of DNA from each isolate was used for RT-PCR reactions.

3.3.2 Antimicrobial Susceptibility Testing

The minimum inhibitory concentration (MIC) of all *N. gonorrhoeae* isolates to ciprofloxacin (Sigma-Aldrich) was assessed by the agar dilution method (25). Antimicrobial susceptibility criteria recommended by the Clinical and Laboratory Standards Institute (CLSI) were used to

Table 3.1 *N. gonorrhoeae* and non-*N. gonorrhoeae* isolates used in this research.

Isolate selection	Organisms	Geographic source	No	References
Panel 1	*Neisseria gonorrhoeae*	Saskatchewan	110	35, 37
		China	96	36
		WHO	10	24
		USA	13	Dillon Culture Collection
		South America and the Caribbean	25	Dillon Culture Collection
		Total	*254*	
	Non-*N. gonorrhoeae*[b]	Canada	13	NML[a]
	Non-*Neisseria* species[b]	Canada	10	NML
		Total	*23*	
	Total isolates		**278**	
Panel 2	*N. gonorrhoeae*	Canada	100	NML
	Non-*N. gonorrhoeae* & non-Neisseria species[c]	Canada	52	NML
	Total isolates		**152**	
Panel 3	*N. gonorrhoeae*	Canada	99	NML
	Non-*N. gonorrhoeae* & non-*Neisseria* species.[b]	Canada	23	NML
	Total isolates		**122**	

[a]National Microbiology Laboratory

[b]*N. animaloris, N. elongate, N. flava, N. lactamica, N. meningitides, N. mucosa, N. perflava, N. polysacchareae, N. sicca, N. subflava, N. wadsworthii, N. weaverii, N. cinerea, E. faecalis, E. faecium, E. coli, K. oxytoca, L. jensenii, M. catarrhalis, P. aeruginosa, S.* Typhimurium, *S. aureus, S. epidermis*

[c]*A. vaginae, B. ureolyticus, C. albicans, C. glucoronolyticum, C.. urealyticum, C. xerosis, C. neoformans, E. aerogenes, E. faecalis, E. faceium, E. coli, G. vaginalis, K. oxytoca, L. crispatus,*

L. gasseri, *L. iners*, *L. jensenii*, *L. buccalis*, *L. monocytogenes*, *M. curtisii*, *M. catarrhalis*, *N. animalis*, *N. animaloris*, *N. cinerea*, *N. elongate*, *N. flava*, *N. flavescens*, *N. lactamica*, *N. meningitidis*, *N. mucosa*, *N. perflava*, *N. polysaccharea*, *N. sicca*, *N. subflava*, *N. wadsworthii*, *N. weaver*, *P. niger*, *P. anaerobius*, *P. bivia*, *P. mirabilis*, *P. aeruginosa*, *S.* Typhimurium, *S. aureus*, *S. epidermidis*, *S. agalactiae*, *S. gordonii*, *S. infantis*, *S. oralis*, *S. pyogenes*, *U. urealyticum*, *U. parvum*, *M. hominis*.

interpret ciprofloxacin MICs as follows: sensitive (≤ 0.06 µg/mL), intermediate (0.125 to 0.50 µg/mL) and resistant (≥ 1.0 µg/mL; 25).

3.3.3 Primers and Real-Time (RT) PCR

Two different forward primers and one common reverse primer with homology to *N. gonorrhoeae gyrA* were designed (United States utility patent #62/088,332) using Primer-BLAST (26). Primer pair *gyrA*-W, for which the forward primer was designed to amplify a conserved region of *gyrA*, comprised forward primer *gyrA*-W-F (5'GCGATTCCGCAGTTTACGA3') and the common reverse primer *gyrA*-R (5'CGAAATTTTGCGCCATACGGACGAT3'). Primer pair *gyrA*-M, for which the forward primer was designed to amplify the region containing the *gyrA* S91 and *gyrA* D95 SNPs associated with ciprofloxacin resistance, comprised forward primer *gyrA*-M-F (5' TACCACCCCCACGGCGATTT3') and the common reverse primer *gyrA*-R.

For panels 1 and 3 RT-PCR was performed using an Applied Biosystems (AB) StepOnePLus RT-PCR system (Life Technologies) on a 96-well plate (Life Technologies) platform and for panel 2 using ViiA 7 RT-D2 RT-PCR Instrument (Life Technologies) on a 96-well plate platform with the SYBR Select Master Mix reagent kit (Life Technologies). PCR reactions contained 5µl of 2X SYBR-Green Master Mix, 0.25µl of each primer (10µM) and 2µl of DNA template (50 ng/µl). The final reaction volume was adjusted to 10µl with deionized water. PCR was conducted according to the manufacturer's guidelines with the following modifications: initial holding and activation at 50°C for 2min followed by a secondary holding at 95°C for 2 min. PCR was performed for 25 cycles at 95°C for 15 sec. and 60°C for 30 sec. The post-PCR melt curve was performed between 60°C to 95°C with 0.3°C temperature increments. DNA from WHO strain F was used as a positive control and a sample without DNA was used as a negative control. Data were collected at 50°C in the holding stage, at 60°C in the annealing stage (for amplification data), and during the melt curve process (for melt curve data).

3.3.4 Multiplex RT-PCR Analyses

For multiplex RT-PCR analyses, reactions contained 5µl of 2X SYBR-Green Master Mix, 0.25µl of each of the forward primers (10µM), 0.25µl of the common reverse primer (10µM) and 2µl of the DNA template (50ng/µl). The final reaction volume was adjusted to 10µl with deionized water.

The method for multiplex RT-PCR was the same as described above. Initially, the sensitivity and specificity of the primers were analyzed using purified DNA from 254 *N. gonorrhoeae* and 23 non-*N. gonorrhoeae* isolates (Table 2.1) using single primer pairs and in a multiplex format. These tests were validated with the same primers and methods but using panel 2 isolates. A subset of DNA from panel 2 isolates was re-validated as panel 3 (Table 2.1).

3.3.5 Calculation of Specificity and Sensitivity

Specificity and sensitivity were calculated using the formulas described before (27).

3.3.6 DNA Sequencing

gyrA genomic region of *N. gonorrhoeae* was PCR amplified with primers *gyrA*-F (ACTGTACGCGATGCACGAGC) and *gyrA*-R (TCTGCCAGCATTTCATGTGAG)[19]. Sanger sequencing of the amplicon was performed at Eurofins Genomics, Louisville KY, USA.

3.4 RESULTS

3.4.1 Specificity and Sensitivity of *gyrA* Primers for Identification of *N. gonorrhoeae* Either in Single PCR or Multiplex Formats

Individually and in multiplex, *gyrA*-W and *gyrA*-M primers did not amplify DNA from any of the non-*N. gonorrhoeae* isolates (Table 3.2) indicating 100% specificity. A non-specific melt curve was produced by the *gyrA*-M primer pair for *Lactobacillus jensenii* that was not reproduced on the repeated analyses. Similarly, the *gyrA*-W primer pair produced non-specific melt curves for *N. animaloris* and *N. meningitidis*, which were not observed in repeated experiments. Non-specific melt curves were considered negative amplification, as these melt curve temperatures fell beyond the expected range and do not interfere with the melt curve interpretation of the ciprofloxacin susceptibility status of *N. gonorrhoeae*. The *gyrA*-W primer pair and *gyrA*-M primer pair amplified 57% and 58% of the 254 *N. gonorrhoeae* isolates respectively (Table 2.2). In the multiplex format with three primers (2 forward and a common reverse primer), all 254 *N. gonorrhoeae* isolates were amplified and no non-*N. gonorrhoeae* isolates were amplified (Table 2.2). Therefore the multiplex assay showed 100% sensitivity and specificity for the identification of *N. gonorrhoeae*.

3.4.2 Multiplex Primers in Ascertaining Ciprofloxacin Susceptibility Status

94 *N. gonorrhoeae* isolates (a subset of panel 1) were analyzed by multiplex RT-PCR; where 47 isolates were previously determined to be ciprofloxacin susceptible (MIC 0.002-0.063ug/mL) and another 47 isolates were determined as ciprofloxacin intermediate/resistant (MIC 0.125-64ug/mL). Putative ciprofloxacin susceptibility status was precisely ascertained by melt curve analyses, which distinctly differentiated between ciprofloxacin susceptible and intermediate/resistant *N. gonorrhoeae* isolates. Susceptible *N. gonorrhoeae* produced melt curve values ~78.0°C and intermediate/resistant *N. gonorrhoeae* produced melt curves values 80.0-83.0°C. Mutations in the *gyrA* sequence resulting in S91 and D95 amino acid alterations were distinguished using the multiplex assay (Figure 3.1A). Further, the multiplex assay performed equally well in differentiating WHO *N. gonorrhoeae* reference strains into ciprofloxacin susceptible and intermediate/resistant groups (Figure 3.1B). The *gyrA* primers in the multiplex format successfully identified WHO-B, C, F, O and P strains as ciprofloxacin sensitive (melt curve ~78.0°C), and WHO-K, L and M strains as intermediate/resistant (melt curve 80.0-83.0°C).

Table 3.2 Sensitivity and specificity of *gyrA*-W and *gyrA*-M primer pairs in multiplex format for identification of *N. gonorrhoeae* isolates (Panel 1) and determination of their ciprofloxacin susceptibility status

	No isolates amplified	**Sensitivity (%)**	**Specificity (%)**
Single *gyrA* primers			
N. gonorrhoeae amplified with *gyrA*-W	144/254	57	NA
Non-*N. gonorrhoeae* amplified with *gyrA*-W	0/23	NA	100
N. gonorrhoeae amplified with *gyrA*-M	148/254	58	NA
Non-*N. gonorrhoeae* amplified with *gyrA*-M	0/23	NA	100
Multiplex *gyrA*-M and *gyrA*-W			
N. gonorrhoeae	254/254	**100**	NA
Non-*N. gonorrhoeae*	0/23	NA	**100**
Ciprofloxacin susceptible[a] *N. gonorrhoeae*	82/82	**100**	99[c]
Ciprofloxacin intermediate/resistant[a] *N. gonorrhoeae*	171/172	**99**	100

[a]As per ciprofloxacin MIC analyses of *N. gonorrhoeae*: 82 isolates susceptible, 7 isolates intermediate, and165 isolates resistant.
[b]NA - not applicable
[c]One ciprofloxacin-resistant isolate was incorrectly identified as ciprofloxacin susceptible

The ciprofloxacin susceptibility status of panel 1 isolates had previously been determined with MIC analyses: 82 isolates were susceptible (MIC 0.002-0.063ug/mL), while 172 isolates were intermediate (n=7; MIC 0.125-0.5ug/mL) or resistant (n=165; MIC 2-64ug/mL). With multiplex RT-PCR, 171/172 isolates were characterized as ciprofloxacin intermediate/resistant and 82/82 isolates were characterized as ciprofloxacin susceptible (Table 3.2). One resistant isolate was incorrectly classified as susceptible after amplification. Thus *gyrA* primers in multiplex are 100% sensitive and 99% specific for the identification of ciprofloxacin susceptible isolates. Similarly, the primers are 99% sensitive and 100% specific in the identification of ciprofloxacin intermediate/resistant *N. gonorrhoeae* isolates (Table 3.2).

3.4.3 Validation of RT-PCR Multiplex Tests for *N. gonorrhoeae* Identification and Ciprofloxacin Susceptibility Status Determination

The *gyrA* primers were used to ascertain gonococcal identification and susceptibility status with panel 2 isolates. Used as single primer pairs, *gyrA*-W was 94% sensitive and 100% specific for the identification of *N. gonorrhoeae*, and *gyrA*-M was 100% sensitive and specific for *N. gonorrhoeae* identification (Table 3.3). Some non-specific peaks were observed with the *gyrA*-M primer pair and interpreted to be non-specific amplifications: short peak melt curves with temperatures 84.12°C, 86.87°C and 87.21°C were observed for *Atopobium vaginae*, *N. animaloris* and *Salmonella* Typhimurium, respectively. *N. perflava* produced a melt curve at 76.65°C for the *gyrA*-W primer pair but this observation was not reproduced in repeated experiments (data not shown).

With panel 3, which comprised DNA from isolates in panel 2, primer pair *gyrA*-W was 93% sensitive and 100% specific for *N. gonorrhoeae* identification while *gyrA*-M was 99% sensitive and 100% specific for *N. gonorrhoeae* identification (Table 3.3). In multiplex analyses, the primers were 100% sensitive and specific for *N. gonorrhoeae* identification. Amplification of *N. animaloris* and *N. meningitides* produced melt curves at 76.65°C for *gyrA*-W but was not reproduced on replication and the result was interpreted as negative amplification.

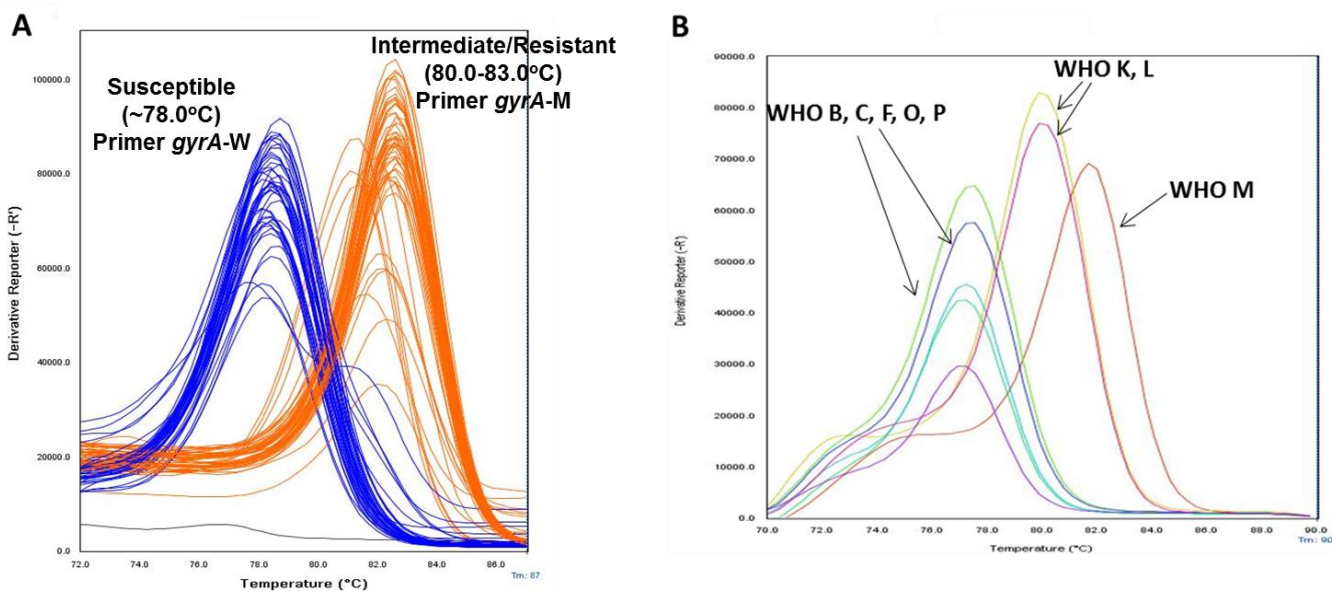

Figure 3.1 Multiplex RT-PCR melt curve representation of ciprofloxacin susceptible and intermediate/resistant *N. gonorrhoeae* isolates. (A) Ciprofloxacin susceptible (n=47) and resistant (n=47) *N. gonorrhoeae* isolates (a subset of panel 1). Blue lines represent susceptible isolates, orange lines represent intermediate/resistant isolates, and black lines represent no template negative control. (B) WHO *N. gonorrhoeae* reference strains (n=8). Strains B, C, F, O, and P are susceptible (melt curve ~78°C) and strains K, L, and M are intermediate/resistant (melt curve 80.0-83.0°C).

Table 3.3 Validation and re-validation of primers *gyrA*-W and *gyrA*-M using *N. gonorrhoeae* and non-*N. gonorrhoeae* isolates in panel 2 and panel 3 by RT-PCR.

	Panel 2			Panel 3[a]		
	No isolates amplified	**Sen[b] (%)**	**Spe[c] (%)**	**No isolates amplified**	**Sen (%)**	**Spe (%)**
Single *gyrA* primers						
N. gonorrhoeae amplified with *gyrA*-W	94/100	94	NA[d]	92/99	93	NA
Non-*N. gonorrhoeae* amplified with *gyrA*-W	0/52	NA	100	0/23	NA	100
N. gonorrhoeae amplified with *gyrA*-M	100/100	100	NA	98/99	99	NA
Non-*N. gonorrhoeae* amplified with *gyrA*-M	0/52	NA	100	0/23	NA	100
Multiplex *gyrA*-M and *gyrA*-W						
N. gonorrhoeae	NP[e]	NP	NP	99/99	**100**	NA
Non-*N. gonorrhoeae*	NP	NP	NP	0/23	NA	**100**
Ciprofloxacin susceptible[f] *N. gonorrhoeae*	NP	NP	NP	47/47	**100**	100
Ciprofloxacin intermediate/resistant[f] *N. gonorrhoeae*	NP	NP	NP	52/52	**100**	100

[a]Primary purpose of Panel 3 was to assess the performance of multiplex primers using a majority of Panel 2 isolates; [b]Sen – Sensitivity; [c]Spe – Specificity; [d]NA – not applicable; [e]NP – not performed; [f]As per ciprofloxacin MIC analyses of *N. gonorrhoeae*: 47 isolates susceptible, 52isolates resistant

3.5 DISCUSSION

A method for the simultaneous identification of the pathogen *N. gonorrhoeae* and its ciprofloxacin susceptibility status has been described. Primer *gyrA*-W forward was designed to include the most conserved region in the *gyrA* sequence that is not common in non-*N. gonorrhoeae* species. The *gyrA*-M forward primer was designed to include the *gyrA* sequence harbouring S91 and D95 mutations. These primers, paired with the common reverse primer, were not anticipated to be highly sensitive when used as individual pairs; however, the multiplex test format had high sensitivity and specificity (>99%) for detecting *N. gonorrhoeae* and its ciprofloxacin susceptibility status in a single amplification.

The *gyrA* and *parC* genomic regions of *N. gonorrhoeae* are associated with ciprofloxacin resistance (19). *parC* mutations in association with *gyrA* mutations maintain higher levels of overall resistance (MIC ≥1) whereas lower levels of resistance are associated with *gyrA* mutations only. On the other hand, *parC* mutations alone are not associated with ciprofloxacin resistance (22, 28, 29). In our research, we considered *gyrA* mutations alone and identified the ciprofloxacin susceptible and resistant isolates in a single multiplex RT-PCR assay. Thus, our results support the findings by others and demonstrate that the *gyrA* region alone is sufficient for the determination of ciprofloxacin susceptibility status (13, 18, 22, 30). We determined, through DNA sequencing, that the majority of intermediately susceptible isolates in our research carried mutations only at the S91 or D95 position, and not both (Table 3.4); one intermediately susceptible isolate in Panel 2 had mutations both at S91 and D95 positions. Similarly, the majority of resistant isolates carried mutations both at S91 (S91F) and D95 positions (D95G, D95A or D95N). One resistant isolate in Panel 1 had a mutation only at the D95 position. Thus, we showed that ciprofloxacin resistance can arise due to mutations at both S91 and D95 amino acid positions, and analysis of S91 position alone is not sufficient for a comprehensive determination of ciprofloxacin susceptibility status.

To our knowledge, this is the first report of simultaneous detection of *N. gonorrhoeae* as well as its ciprofloxacin susceptibility status in a single and simple test that is highly specific, sensitive, inexpensive, and rapid. The costs associated with culturing, training of laboratory personnel, as well as the current costs of NAATs, are beyond the economic capabilities of many resource-limited

settings. The cost/sample in the RT-PCR assay discussed in our test ($11.07) is about two times less than the costs/sample incurred by the current *N. gonorrhoeae* diagnostic NAATs or the costs associated with SNP and/or ID testing at the NML, estimated at $75/sample including labour. Thus the assay discussed in our work is far more affordable than the current NAATs for *N. gonorrhoeae* diagnosis. One study demonstrated the detection of *N. gonorrhoeae* and its ciprofloxacin susceptibility status, but this assay required three consecutive tests for a clear interpretation (31).

Several recent publications introduced RT-PCR based methods for the detection of mutations at the *gyrA* S91 locus from clinical specimens (18, 30, 32). However, these analyses were restricted only to the S91 locus and the assays could be more likely to have errors if ciprofloxacin resistance arose through mutations in the D95 locus. In our work, we have observed through amplicon sequencing, ciprofloxacin resistance primarily arose due to mutations in both S91 and D95 loci, while intermediate resistance mainly arose due to mutations in either S91 or D95 loci. (Table 3.4).

Although the results of this research highlight the utility of this multiplex RT-PCR method in identifying *N. gonorrhoeae* and determining its ciprofloxacin susceptibility status, caution is needed as only a very limited number of non-*N. gonorrhoeae* isolates were tested. In addition, further studies are needed to investigate if the assay has clinical applicability by testing clinical specimens directly with the aim of personalized care (18).

Our findings show promise for the development of a POCT for the identification of *N. gonorrhoeae* and its ciprofloxacin susceptibility status. POCTs facilitate the diagnosis at the health care provider's office and avoids unnecessary treatment delays. This eliminates the need for patients to return for post-diagnosis treatments and prevents unwanted complications and disease transmission (31). Given its probable low cost and relative ease of use, this assay could have major implications for resource-poor, high disease burden countries (33, 34). Further, our assay may prove helpful to clinicians and laboratories to respond to infectious disease control organizations, increase surveillance for ciprofloxacin resistance, and present coherent findings for epidemiological and surveillance purposes. With the proper diagnosis of the AMR status, the use of ciprofloxacin can be increased from 8% (with NAAT based diagnosis) to over 81% (with *gyrA* based diagnosis), and thereby reduce the use of last resort antimicrobials (18). Thus, a rapid and

Table 3.4 Single Nucleotide Polymorphisms (SNPs) observed at S91 and D95 amino acid positions of *N. gonorrhoeae* isolates based on *gyrA* amplicon sequencing.

		Susceptible	Intermediate			Resistant				Total Tested
SNP		S91/D95	S91/D95G	S91F/D95	S91Y/D95	S91/D95N	S91F/D95G	S91F/D95A	S91F/D95N	
Panel 1[a]	No Isolates	35	1	2	2	1	44	56	12	154
	Susceptibility Status	S[b]	I[b]	I	I	R[b]	R	R	R	
Panel 2	No Isolates	46	-	2	1	-	43	5	3	100
	Susceptibility Status	S	-	I	I	-	I, R[c]	R	R	

[a]*gyrA* sequencing information from Panel 1 is presented for 154/254 isolates. Sequencing information is not available for older isolates. Ciprofloxacin susceptibility status of Panel 1 and Panel 2 isolates was confirmed with MIC testing
[b]S – Susceptible; I – Intermediate; R – Resistant
[c]1/43 isolate intermediate and 42/43 isolates resistant

reliable assay, such as the platform discussed by our work, would improve the understanding of the extent of the disease worldwide.

3.6 ACKNOWLEDGEMENTS

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CHAPTER 4: ENHANCED SURVEILLANCE OF *NEISSERIA GONORRHOEAE* AND ITS CIPROFLOXACIN SUSCEPTIBILITY PROFILE THROUGH CULTURE INDEPENDENT POINT-OF-CARE TESTING

4.0 INTERFACE

This chapter expands research described in Chapter 3, where a test comprising of multiplex primers for the simultaneous identification of *N. gonorrhoeae* and its ciprofloxacin susceptibility status was assessed using DNA from *N. gonorrhoeae* cultures. In this research, the performance of the test was further evaluated using different types of clinical specimens (i.e. remnant Aptima urine specimens, DNA from urines and raw urines). Susceptibility testing on remnant Aptima specimens will serve as a supplementary test for characterizing the resistance profile of a diagnosed *N. gonorrhoeae* isolate. As the goal is to introduce the test to local health centers, assessment with clinical specimens highlights the strengths and weaknesses of the test, and indicate the best specimen type for this test. The work undertaken in this chapter was presented at the Saskatchewan Epidemiology Association 19th Annual Fall Symposium and Workshop held in Regina, Saskatchewan, Canada, on October 8-9th, 2019.

Sumudu R Perera conceptualized the research with guidance from Dr. Dillon, carried out laboratory experiments, analyzed data, wrote the manuscript and supervised Kristen M Mitzel, who worked on this project as a summer student and an undergraduate honours student.

Kristen M Mitzel carried out laboratory experiments and analyzed data as part of an undergraduate honours project and a Summer research project.

Jo-Anne R Dillon conceptualized the research, provided guidance with the experimental design, feedback and editing on the manuscript and held the research grant that supported this work.

4.1 ABSTRACT

With the emergence of the post-antibiotic era, multidrug-resistant *N. gonorrhoeae* has become an imminent threat, throughout the world. With time running out on the use of existing antimicrobials,

it is of vital importance to find new strategies to combat the emergence and spread of drug-resistant *N. gonorrhoeae*. We developed a PCR assay using multiplex primers (*gyrA*-W-F, *gyrA*-M-F, and *gyrA*-R) that are unique for *N. gonorrhoeae* and can ascertain its ciprofloxacin susceptibility status in a single multiplex RT-PCR reaction. In this research, the feasibility of using this molecular test on urines and remnant Aptima urine specimens for antimicrobial susceptibility prediction was assessed. This test, with remnant Aptima urine specimens (n=83), had an overall 71% sensitivity for *N. gonorrhoeae* identification; where 92% and 53% sensitivities were observed for ciprofloxacin susceptible and resistant specimens, respectively. When DNA extracted from urine specimens was used, 80% sensitivity and specificity were observed for *N. gonorrhoeae* identification. The primers were also assessed on a hydrogel platform for their ability to identify ciprofloxacin susceptibility status of gonococcal isolates in this setup and had demonstrated 100% sensitivity and specificity. The development of rapid diagnostic tests for *N. gonorrhoeae* and more importantly for its ciprofloxacin susceptibility prediction will open possibilities of using no-longer recommended older antimicrobials in therapy and in-turn promote the preservation of cherished last resort antimicrobials.

4.2 INTRODUCTION

N. gonorrhoeae is a significant global public health concern, with 86.9 million reported new infections annually (1). Over time, gonococci have developed resistance to every class of antibiotic introduced for therapy, including third-generation cephalosporins, the last class of antibiotic used in single-dose therapy (2-4). The recommended treatment in many countries now comprises co-therapy with 1g of oral azithromycin and 250mg of intramuscular ceftriaxone (5). Nonetheless, there have been reports of gonococci with decreased susceptibility to azithromycin and/or ceftriaxone (6-9), as such many countries in the European Union, only recommend ceftriaxone or cefixime for treatment of gonorrhea (10). As we have a limited pool of antibiotics to combat the emerging multi-drug resistant *N. gonorrhoeae,* there is an increased urgency for new strategies to treat gonococcal infections.

Early diagnosis of infection is vital for proper control of gonorrhea (11, 12). The identification of *N. gonorrhoeae* in high-income countries is usually accomplished by nucleic acid amplification tests (NAATs; 13). A limitation of NAATs is that the antimicrobial susceptibility (AMS) of *N. gonorrhoeae* positive specimens cannot be completed at present, as such testing requires the pathogen to be cultured. Due to the widespread use of NAATs, AMS testing of *N. gonorrhoeae* isolates has significantly declined over the last decade (14). In resource-poor settings, patients are treated empirically based on symptoms (15, 16), either due to costs associated with diagnostic tests or the unavailability of appropriate laboratory facilities. In these instances, syndromic management often fails to identify asymptomatic infections or resistant isolates (17, 18).

An important factor contributing to the rising prevalence of gonorrhea is treatment delay during which patients, waiting for a positive diagnosis, can spread the disease to their contacts (19). At times, despite the availability of laboratory facilities, the delays in reporting results (which can take up to days) severely hinder timely treatment (11). It has been reported that a rapid test with a sensitivity as low as 63% can significantly increase the number of patients treated, instead of waiting for a highly sensitive, yet slower test (20, 21).

Ciprofloxacin, a fluoroquinolone antibiotic, was widely been used for treating *N. gonorrhoeae*

infections from the mid-1980s onwards (3). The World Health Organization (WHO) recommends the discontinuation of an antibiotic when resistance is observed in >5% of isolates tested (5). In Canada, between 2004 and 2014, ciprofloxacin resistance in *N. gonorrhoeae* increased from 6.3% to 34% of isolates tested and is no longer recommended for the primary treatment of gonorrhea (21). However, in many parts of the world, a high percentage of isolates remain susceptible to ciprofloxacin, i.e. Saskatchewan 90%, United States 80.4%, Europe 66%, and Australia 73% (22-25). Thus if ciprofloxacin susceptible isolates could be identified, ciprofloxacin can be re-introduced for treatment of these isolates.

Recently a commercial NAAT test ascertaining gonococcal ciprofloxacin susceptibility status was licensed in the United States (26). However, this assay only targets the *gyrA* S91F amino acid substitution. Furthermore, many other tests designed to identify ciprofloxacin susceptible *N. gonorrhoeae* isolates have also only focused on the *gyrA* S91 position (27-31). We and others have previously shown that there can be other substations at the S91 position (i.e. S91Y), as well as substitutions at the D95 amino acid position which confer resistance to ciprofloxacin (32-34). Thus focusing on the S91 position alone may limit the ability to identify all susceptible isolates. Consequently, still there is an urgent need for a comprehensive test that can identify those infected with ciprofloxacin susceptible *N. gonorrhoeae* isolates, and preserve vital last resort antimicrobials.

A reliable and rapid point-of-care (POC) diagnostic test, capable of AMS testing in *N. gonorrhoeae* would be a critical advancement in the diagnostic capability, especially given the limitations of current NAATs for AMS testing (35). Furthermore, it was shown that targeted ciprofloxacin therapy may be cost-effective in comparison to current dual drug therapy when the frequency of testing and the prevalence of resistance is accounted for (36). Previously with *N. gonorrhoeae*-specific multiplex primers and DNA from *N. gonorrhoeae* cultures, we obtained over 99% sensitivity and specificity for identifying ciprofloxacin susceptible isolates (Chapter 3: 31, 37). In this research, we assessed the efficacy of these multiplex primers in identifying *N. gonorrhoeae* and predicting ciprofloxacin susceptibility from urines and remnant Aptima urine specimens.

4.3 MATERIALS and METHODS

4.3.1 Clinical Specimens and Bacterial Strains

Two types of specimens were used; urines and remnant Aptima urine specimens. The 331 remnant Aptima urine specimens were obtained from the Roy Romanow Provincial Laboratory (RRPL), Regina SK. The specimens were collected between 2015 and 2016. *N. gonorrhoeae* was identified using the Aptima Combo 2 assay (Hologic Gene-Probe Inc., San Diego CA) at the RRPL. Of the 331 specimens, 301 were *N. gonorrhoeae* positive and 30 were *N. gonorrhoeae* negative. Also, 20 *N. gonorrhoeae* positive and 20 *N. gonorrhoeae* negative urines were obtained from the Saskatchewan Health Authority (SHA), Regina region (Regina SK). These specimens had been tested for *N. gonorrhoeae* using the Aptima Combo 2 assay. The DNA extracted from urines and raw urines (without DNA extraction) was used in the analyses. For experiments involving urines, positive controls consisted of DNA from the WHO *N. gonorrhoeae* reference isolates F, P, G, K and N (38), while the negative controls included DNA from *Neisseria weaveri*, *Neisseria wadsworthii*, *Klebsiella oxytoca*, *Staphylococcus aureus* and *Lactobacillus iners*, obtained from the National Microbiology Laboratory (NML), Winnipeg MB.

The portable hydrogels were manufactured by the Aquila Diagnostic Systems Inc. Edmonton AB, Canada. This hydrogel-based diagnostic platform utilizes Real-Time Polymerase Chain Reaction (RT-PCR) melt curve analysis to amplify nucleic acids directly from clinical specimens and report the presence of specific genetic targets. For experiments using hydrogels DNA from the following isolates and specimens were used; *N. gonorrhoeae* 971008 and *N. gonorrhoeae* C0910B027331 (10ng/µl; Dillon culture collection), and DNA from urine #54 (10ng/µl) and urine #55 (2.6ng/ul; SHA, Regina Region). DNA concentrations of raw urines were not measured since urines are highly diluted.

4.3.2 DNA Extraction

N. gonorrhoeae isolates were stored at -80°C in brain heart infusion medium (Difco BD Bioscience) with 20% glycerol. Aliquots from frozen inocula were cultured on GC medium base (Difco BD Bioscience) supplemented with 1% Kellogg's defined supplement. Plates were incubated at 35-37°C with 5% CO_2 in a humid environment for 18 to 24h (39).

Remnant Aptima urine specimens and urines were shipped on dry ice to the Dillon laboratory. Aptima specimens were stored at 4^{o}C for 2-3 days prior to DNA extraction, while the urines were stored at -80^{o}C until DNA extraction.

DNA extraction from bacterial cultures and remnant Aptima urine specimens was performed using a QIAamp DNA Mini kit (Qiagen) according to the manufacturer's instructions. DNA from bacterial cultures was quantified using NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) and was normalized to 50ng/µl. Due to their variability, DNA concentrations were not standardized for remnant Aptima urine specimens.

Urine specimens were spun at 13,000xg for 10 min. The resulting pellets were treated overnight with Proteinase K and ATL buffer. On the following day, DNA was extracted using a QIAamp DNA Mini kit (Qiagen) according to the manufacturer's protocol. DNA was quantified using a Qubit 4 Fluorometer (Thermo Fisher Scientific) and was normalized to 50ng/µl for all the positive and negative control specimens.

4.3.3 DNA Sequencing

To determine ciprofloxacin susceptibility status of *N. gonorrhoeae*, a fragment of the *gyrA* genomic region of *N. gonorrhoeae*, corresponding to the S91 and D95 amino acid positions, was PCR amplified from *N. gonorrhoeae* cultures, remnant Aptima urine specimens, and urines with primers *gyrA*-F (ACTGTACGCGATGCACGAGC) and *gyrA*-R (TCTGCCAGCATTTCATG TGAG) (40). Sanger sequencing of the amplicons was performed at Eurofins Genomics (Louisville KY, USA).

4.3.4 Multiplex Primers

Two different forward primers and a common reverse primer with homology to *N. gonorrhoeae gyrA* were designed (United States utility patent #62/088,332) using Primer-BLAST as reported previously (Chapter 3, 32, 41). Briefly, primer pair *gyrA*-W, for which the forward primer was designed to amplify a conserved region of *gyrA*, comprised the forward primer *gyrA*-W-F (5'GCGATTCCGCAGTTTACGA3') and the common reverse primer *gyrA*-R (5'CGAAATTTT GCGCCATACGGACGAT3'). Primer pair *gyrA*-M, for which the forward primer was designed

to amplify the region containing the *gyrA* SNPs associated with ciprofloxacin resistance, comprised the forward primer *gyrA*-M-F (5' TACCACCCCCACGGCGATTT3') and the common reverse primer *gyrA*-R (32).

4.3.5 Real-Time (RT) PCR

RT-PCR was performed using an Applied Biosystems StepOnePLus instrument (Life Technologies) on a 96-well plate platform (Life Technologies) with the SYBR Select Master Mix reagent kit (Life Technologies). PCR reactions contained 5µl of 2X SYBR-Green, 3µl of combined primer mix (10µM) and 2µl of DNA template (at various concentrations). PCR was performed with an initial holding at 50°C for 2min followed by a secondary holding at 95°C for 2 min. PCR was performed for 25 cycles at 95°C for 15 sec and 60°C for 30 sec. The post-PCR melt curve was determined between 60°C to 90°C, with a 0.3°C temperature increments. Each experiment was performed in duplicate. The sensitivity and specificity of the melt curve predicted ciprofloxacin susceptibility status was calculated using the formulas described before (40).

For experiments with hydrogels, PCR reactions contained 0.25µl of each primer (which were either at 5, 10 or 15µM), and 2µl of DNA template (at various concentrations). The DNA concentration was not adjusted to all samples, due to variability in urines. The final reaction volume was adjusted to 10µl with deionized water. PCR was conducted as; initial holding at 50°C for 2 min followed by a secondary holding at 95°C for 2 min. Amplification was performed for 40 cycles (experiment 1) or 25 cycles (experiment 2) at 95°C for 15 sec and 60°C for 30 sec. The 25 and 40 PCR cycles were determined with preliminary experiments involving urines and previous observations (32). The post-PCR melt curve was determined between 60°C to 95°C with 0.3°C increments. All experiments were performed in duplicates.

4.3.6 Hydrogels

Hydrogels were manufactured by Aquila Diagnostic Systems Inc., (Edmonton AB). Different SYBR-Green dye concentrations (2X, 3X, 4X, 5X and 6X) were incorporated into the gels before shipping to the Dillon laboratory. Primers and DNA were added to the gels immediately before PCR amplification.

4.4 RESULTS

4.4.1 Predictability of Ciprofloxacin Susceptibility Status using Aptima Urine Specimens

From the 301 *N. gonorrhoeae* positive remnant Aptima urine specimens, 146 (49%) specimens were correctly identified as *N. gonorrhoeae* positive, and from the 30 *N. gonorrhoeae* negative specimens, 21 (70%) were identified as negative. From the 146 specimens identified as *N. gonorrhoeae* positive, RT-PCR based melt curve analyses predicted 105 were ciprofloxacin susceptible and 41 were intermediate or resistant.

To ascertain the accuracy of RT-PCR based prediction of ciprofloxacin susceptibility status using the *gyrA* multiplex primers, 83 specimens were amplicon sequenced for the region surrounding the *gyrA* S91 and D95 positions. The results from sequencing were compared to melt curve based predictions of ciprofloxacin susceptibility status (Table 4.1). Of the 83 specimens, 54 was identified as susceptible and 29 was identified as resistant with melt curve analysis. However, sequence analysis indicated that 16 resistant isolates were incorrectly recognized as susceptible and these 16 isolates had the S91F/D95G substitutions. Of the 45 resistant isolates, 35 carried the S91F/D95G, 9 carried the S91F/D95A, and 1 carried the S91F/D95 substitutions.

These results were indicative that although resistance cannot be predicted with a high sensitivity, ciprofloxacin susceptible isolates can be predicted, with over 90% accuracy, using DNA from remnant Aptima urine specimens. However, the results should be taken with caution, as 16 resistant isolates were also identified as susceptible.

4.4.2 Sensitivity of the *gyrA* Multiplex Primers When Using Urines

Next, the primers were assessed using urines. When using DNA from *N. gonorrhoeae* and non-*N. gonorrhoeae* cultures, sensitivity and specificity of 100% were observed (Table 4.2). With DNA from *N. gonorrhoeae* positive urines, 80% sensitivity was observed, while a 15% sensitivity was noted with *N. gonorrhoeae* positive raw urines (i.e. no DNA extraction step was performed). Similarly, an 80% specificity was observed with DNA from *N. gonorrhoeae* negative urines, and a 100% specificity was observed with *N. gonorrhoeae* negative raw urines.

Table 4.1 The ciprofloxacin susceptibility status predicted with melt curve analysis[a]

	No of specimens	**Susceptibility/Resistance based on Sequencing**	**SNPs**
Susceptible	54	38	S91/D95
Resistant	29	45[a]	S91F/D95G – 35 S91F/D95A – 9 S91F/D95 – 1

[a]16 resistant isolates were identified as susceptible. All these isolates had S91F/D95G substitution

Table 4.2 Sensitivity and specificity of *gyrA* multiplex primers for identification of *N. gonorrhoeae* and its ciprofloxacin susceptibility prediction using urine specimens.

	Sensitivity	**No. Cipro Susceptible[a]**	**No. Cipro Resistant[a]**
DNA from *N. gonorrhoeae* cultures (n=5)	100% (5/5)	2/5	3/5
DNA from *N. gonorrhoeae* positive urines (n=20)	80% (16/20)	16/20[b]	0/20
N. gonorrhoeae positive raw urines (n=20)	15% (3/20)	-	-
	Specificity		
DNA from non-*N. gonorrhoeae* cultures (n=5)	100% (0/5)[c]	-	-
DNA from *N. gonorrhoeae* negative urines (n=20)	80% (4/20)[c]	-	-
N. gonorrhoeae negative raw urines (n=20)	100% (0/20)[c]	-	-

[a]Ciprofloxacin susceptibility status predicted with RT-PCR was verified with *gyrA* amplicon sequencing
[b]Four susceptible specimens had non-specific melt curves
[c]Specificity was determined by the number of non-amplified specimens

Ciprofloxacin susceptible control strains (WHO F and P) were correctly identified as susceptible (2/5; Table 4.2), and the ciprofloxacin intermediate and resistant strains (WHO G, K and N) were correctly identified as resistant (3/5). As *N. gonorrhoeae* positive raw urines and DNA from *N. gonorrhoeae* positive urines represent the same samples, susceptibility status was only determined for DNA samples. Here, 16/20 specimens were identified as ciprofloxacin susceptible by melt curve analysis and were later confirmed by amplicon sequencing the region surrounding the *gyrA* S91 and D95 positions. The four remaining specimens had non-specific melt curves. Thus, with DNA from urines, none of the ciprofloxacin susceptible isolates were incorrectly identified as resistant.

4.4.3 Hydrogel Experiments with *gyrA* Multiplex Primers

We previously used a hydrogel-based RT-PCR detection platform and a panel of *N. gonorrhoeae* specific diagnostic primers to identify *N. gonorrhoeae* DNA from cultures (32). To optimize conditions in hydrogels for the *gyrA* multiplex primers, we evaluated DNA from *N. gonorrhoeae* cultures and *N. gonorrhoeae* positive urines, as well as raw urines using the *gyrA* multiplex primers. The criteria for a positive test consisted of a copy number (Ct) value above the threshold, a melt curve (Tm) at the correct temperature (susceptible 77-78^{o}C and resistant 80-82^{o}C) and visual inspection of the melt curve for a characteristic peak.

In the first experiment (Exp 1, 40 PCR cycles), the best performance was observed with 10µM primer and 2X, 3X, 4X and 5X SYBR-dye, where all 6 *N. gonorrhoeae* specimens amplified and the negative control did not amplify (Table 4.3). DNA from *N. gonorrhoeae* cultures amplified with all SYBR dye and primer concentrations except for two instances; Ng C0910B027331 with 4X SYBR/5µM primer and Ng 971008 with 3X SYBR/5µM primer. DNA from urine #54 amplified under all conditions expect with 2X SYBR/5µM primer. DNA from urine 55 failed to amplify with 2X SYBR, 3X SYBR, and 6X SYBR when15µM primer was used. However, DNA extracted from urine specimens performed best with 4X and 5X SYBR concentrations. The results using raw urines were optimal with 3X SYBR and all three primer concentrations.

In the second experiment (Exp 2, 25 PCR cycles), none of the negative controls amplified, as expected (Table 4.3). However, none of the raw urines or DNA extracted from the urine specimens

Table 4.3 Summary of the hydrogel-*gyrA* multiplex primer experiment. Exp 1 – experiment 1 performed with 40 RT-PCR cycles; Exp 2 – experiment 2 performed with 25 RT-PCR cycles.

Primer Conc.	SYBR Dye Conc.	Isolates/Specimens						
		DNA extracted from *N. gonorrhoeae* cultures		DNA extracted from urines		Raw urines		*N. polysacharea* (negative control)
		C0910B027331 (R)[a]	971008 (S)[b]	#54 (R)	#55 (S)	#54 (R)	#55 (S)	
		Exp 1/Exp2[c]	Exp 1/Exp2	Exp 1/Exp2	Exp 1/Exp2	Exp 1/Exp2	Exp 1/Exp2	Exp 1/Exp2
5 uM	2x	+/+	+/-	**-/-**	+/-	**-/-**	+/-	+/-
	3x	+/+	**-/-**	+/-	+/-	+/-	+/-	**-/-**
	4x	- /+	+/-	+/-	+/-	**-/-**	+/-	+/-
	5x	+/+	+/-	+/-	+/-	+/-	**-/-**	+/-
	6x	+/-	+/-	+/-	**-/-**	**-/-**	**-/-**	**-/-**
10 uM	2x	+/+	+/-	+/-	+/-	+/-	+/-	**-/-**
	3x	+/+	+ /+	+/-	+/-	+/-	+/-	**-/-**
	4x	+/+	+ /+	+/-	+/-	+/-	+/-	**-/-**
	5x	+/+	+/-	+/-	+/-	+/-	+/-	**-/-**
	6x	+/+	+/-	+/-	+/-	**-/-**	+/-	+/-
15 uM	2x	+/+	+ /+	+/-	**-/-**	+/-	**-/-**	+/-
	3x	+/+	+ /+	+/-	**-/-**	+/-	+/-	+/-
	4x	+/+	+ /+	+/-	+/-	+/-	+/-	+/-
	5x	+/+	+ /+	+/-	+/-	+/-	+/-	+/-
	6x	+/+	+ /+	+/-	+/-	**-/-**	+/-	+/-

[a]R – ciprofloxacin resistant
[b]S – ciprofloxacin susceptible

amplified either. Both Ng C0910B027331 and Ng 971008 amplified with 2X SYBR/15µM primer, 3X SYBR/10µM and 15µM primer, 4X SYBR/10µM and 15µM primer, and 5X SYBR/15µM primer concentrations.

Overall, the samples performed the best with 10µM primer concentration, 5X SYBR-Green concentration and 40 PCR cycles. Surprisingly, *N. polysacharea* amplified 9/15 times, and irrespective of the SYBR concentration or the primer concentration (Table 4.3). Subsequent testing with *N. gonorrhoeae* and ddH_2O from different sources indicated that 40 PCR cycles were causing non-specific amplification and was not observed when 25 PCR cycles were used. However, 25 PCR cycles had compromised the amplification of raw urines and DNA from urine specimens. The SYBR dye concentration did not affect the performance of the samples, although 15µM primer concentration worked the best with DNA extracted from *N. gonorrhoeae* cultures (Table 4.3). In this preliminary experiment, DNA extracted from *N. gonorrhoeae* cultures and urines, as well as raw urines had 100% sensitivity for *N. gonorrhoeae* identification and ciprofloxacin susceptibility prediction, with 10µM primer, 5X SYBR-Green and 40 PCR cycles, in the hydrogel assay. (Table 4.3).

4.5 DISCUSSION

This research assesses the feasibility of using urine specimens for the diagnosis of *N. gonorrhoeae* and characterization of its ciprofloxacin susceptibility status with unique multiplex primers (32, 36). Our results shed light on the limitations associated with using remnant Aptima urine specimens for AMS prediction by molecular techniques, as these specimens were not ideal for predicting resistance. However, we observed over 90% sensitivity in susceptibility predictions, but the specificity was low as 42% resistant isolates were also identified as susceptible. Although *N. gonorrhoeae* was not readily amplified from raw urines, the DNA extracted from urines have higher sensitivity for *N. gonorrhoeae* identification and ciprofloxacin AMS prediction than with remnant Aptima urine specimens. Despite the use of *N. gonorrhoeae* positive remnant Aptima specimens in the past for PCR amplification of resistant determinants (42), and evaluation of diagnostic assays (28, 43), these experiments were limited in either time for results (41) or capability for a comprehensive analysis of all markers associated with ciprofloxacin resistance (27, 28, 43, 44).

The *gyrA* and *parC* loci of *N. gonorrhoeae* are associated with ciprofloxacin resistance (45). Although *parC* mutations in association with *gyrA* mutations are implied in higher levels of ciprofloxacin resistance (MIC of ≥1), *gyrA* mutations alone are associated with lower levels of resistance. In contrast, *parC* mutations alone are not associated with ciprofloxacin resistance (31, 46). Thus, in our research, we have further supported that the characterization of *gyrA* alone is sufficient to identify ciprofloxacin susceptible isolates both using DNA from cultures and urines (31, 32, 34, 44, 47). However, in contrary to other PCR based methods for detecting *gyrA* S91 substitutions (44, 47, 48), previously we have shown that reduced susceptibility can arise due to substitutions either at S91 or D95 amino acid positions (32). Therefore, the analysis of the S91 position alone is not sufficient for a comprehensive determination of ciprofloxacin susceptibility status, and our primers are unique to identify resistance arising through mutations corresponding to both S91 and D95 amino acid positions.

Despite the assay's benefits, this research has limitations. Here we only tested a limited number of urine specimens and have not tested other types of clinical specimens, such as swabs. For this

assay to be an ideal POCT, the volume of urine and the type of specimen should be optimized such that the concentration of *N. gonorrhoeae* DNA in the final reaction can be increased (27). Additionally, the minimum inhibitory concentrations (MICs) were not ascertained for any of the specimens because they were derived from remnant Aptima urine specimens. This may be more feasible with swab specimens (29). Due to the limited number of samples and replicates involved with hydrogel experiments, it was difficult to establish the sensitivity and specificity of the *gyrA* primers in the hydrogel platform. Larger sample sizes would allow for more reliable sensitivity and specificity predictions. Furthermore, the number of PCR cycles should be optimized to amplify the urine specimens, but not the DNA from commensal *Neisseria* species.

Despite these limitations, our findings show promise in using DNA extracted from urines to diagnose *N. gonorrhoeae* infections and determine the isolate's ciprofloxacin susceptibility status. Furthermore, this is the first research using direct clinical specimens (i.e. without a DNA extraction step) for molecular AMS testing for ciprofloxacin in *N. gonorrhoeae*; whereas other tests using clinical specimens have utilized a DNA extraction step (27-29). Given that local communities exist with ciprofloxacin susceptible gonococcal isolates (23), our assay enables rapid (~2h) and timely determination of AMS and thereby facilitate the use of no longer recommended antibiotics to treat gonorrhea and promote the preservation of last resort antimicrobials such as cephalosporin and azithromycin (44). As such, this platform shows promise in the development as a POCT for *N. gonorrhoeae* diagnosis and characterization of its ciprofloxacin susceptibility status. POCTs facilitate timely diagnosis at local health clinics and evade unnecessary treatment delays (49). Given its probable low cost and relative ease of use, this assay could have major implications for resource-limited, high disease burden communities.

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CHAPTER 5: CHARACTERIZATION OF ANTIMICROBIAL RESISTANCE GENES FROM *NEISSERIA GONORRHOEAE* POSITIVE REMNANT APTIMA URINE SPECIMENS

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5.0 INTERFACE

Although *N. gonorrhoeae* isolates from different communities can remain susceptible to ciprofloxacin, more and more gonococcal isolates are acquiring mutations leading to multi-drug resistance. Thus, for antimicrobial stewardship and to help in treatment, it is crucial to characterize emerging drug resistance trends and overall resistance prevalence in *N. gonorrhoeae* isolates. This research assessed the use of remnant Aptima urine specimens to characterize eight genes associated with antimicrobial resistance in *N. gonorrhoeae* and to predict antimicrobial resistance/susceptibility to six antibiotics. We reported emerging gonococcal resistance, in Saskatchewan, to azithromycin and cefixime, two of the last resort antimicrobials presently used for gonococcal treatment. Especially, we identified an emerging clone implicated globally in cefixime resistance with *penA* type XXXIV. Thus, this research highlights the importance of molecular analysis of clinical specimens for the prediction of present and emerging AMR in gonococci. This chapter was previously published in *Future Microbiology* DOI:10.2217/fmb-

2019-0161 and is reprinted here with permission. The work discussed in this chapter has been presented at the Gordon Research Conference on Drug Resistance held in Smithfield, Rhode Island, the United States of America, on July 22-27th, 2018, and at the International Union Against Sexually Transmitted Infections (IUSTI) World + European Congress held in Dublin, Ireland on June 27-30th, 2018.

Nidhi R Parmar carried out laboratory experiments, analyzed data and wrote the manuscript

Sumudu R Perera carried out laboratory experiments, performed preliminary data analysis, supervised Jin Wang and provided input on the manuscript

Jin Wang carried out laboratory experiments and performed preliminary data analysis

Paul N Levett and **Jessica Minion** supplied the remnant Aptima urine specimens

Jo-Anne R Dillon conceptualized the research, provided guidance with the experimental design, feedback and editing on the manuscript and held the research grant that supported this work

5.1 ABSTRACT

To ascertain antimicrobial resistance and strain types (STs) of *Neisseria gonorrhoeae* from 50 remnant Aptima urine specimens using molecular methods, mutations predictive of resistance to six antibiotics were identified in eight genes. STs were determined using NG-MAST and NG-STAR. All eight antimicrobial resistance genes could be characterized in 36 specimens. A total of 17 specimens were predicted to be susceptible to all antibiotics, including ceftriaxone. Decreased susceptibility to cefixime and ciprofloxacin resistance was predicted in 11 specimens (PBP2 type 34.001). Overall, 38/50 specimens were predicted to be ciprofloxacin susceptible; 3 were azithromycin resistant. Nineteen NG-MAST and 21 NG-STAR STs were noted. Molecular analysis of remnant Aptima specimens enabled the prediction of emerging gonococcal cefixime and azithromycin resistance, which would otherwise have been undetected.

5.2 INTRODUCTION

Gonorrhea, caused by the pathogen *N. gonorrhoeae*, is the second most common sexually transmitted infection (STI) in Canada (1). There are 78 million new infections of gonorrhea per year worldwide (2012 estimates) (2). Gonorrhea is managed with a combination of case finding, microbiological diagnosis, and treatment with antibiotics. There are no vaccines for this disease. *N. gonorrhoeae* has sequentially developed resistance to every class of antimicrobial agents used for treatment since the introduction of sulphonamides in 1935 (3). Because of this resistance, treatment options for gonorrhea infections are limited and, in many countries, a combination of ceftriaxone, a third-generation cephalosporin and the last class of drug used in monotherapy, with azithromycin is recommended for therapy (4). Increasing resistance to both ceftriaxone and azithromycin, and documented treatment failures to both antibiotics have further intensified the need to find either new treatment modalities, or to steward currently available antibiotics for the treatment of gonorrhea more effectively (5, 6). The World Health Organization (WHO) established a global action plan to control the emergence and spread of antimicrobial resistance (AMR) in *N. gonorrhoeae* with AMR surveillance being a critical component of the plan (4, 7).

AMR determination of *N. gonorrhoeae* isolates requires that the microorganism be cultured so that its antimicrobial susceptibility (AMS) can be ascertained. This is not performed in many regions. Resource-rich settings use nucleic acid amplification tests (NAATs) for the identification of *N. gonorrhoeae* from clinical specimens; the microorganism cannot be cultured for AMR testing from such specimens (8). Resource-limited settings often do not culture for *N. gonorrhoeae* and, in the absence of adequate laboratory facilities, patients rely on syndromic management and empiric treatment of gonococcal infections (3). Thus, the true extent of AMR in *N. gonorrhoeae* is unknown in many areas. One solution to this challenge would be to devise reliable molecular methods for AMR determination or prediction.

Resistance to antibiotics in *N. gonorrhoeae* isolates is caused by mutations in various genes and/or horizontal gene transfer (9). For example, penicillin resistance can be mediated by a plasmid-encoded TEM β-lactamase (bla_{TEM}) or by mutations in several chromosomal genes (10). The stepwise acquisition of mutations in genes such as *penA* (encodes PBP2), *mtrR* (encodes MtrR),

and *porB* (encodes PIB) lead to a reduced affinity for penicillin by PBP2, increased efflux pump activity and membrane impermeability (10). These mutations, with an additional mutation in *ponA* (encodes PBP1), contribute to stepwise increases in the Minimum Inhibitory Concentration (MIC) and ultimately chromosomally-mediated penicillin resistance (10). In addition to these mutations, mosaic patterns (~60 mutations) in the transpeptidase domain of PBP2 can lead to reduced susceptibility or resistance to extended-spectrum cephalosporins (ESCs) (11-14). Resistance to tetracycline is conferred by a mutation in ribosomal protein S10 (encoded by *rpsJ*), which increases gonococcal MICs to tetracycline (MIC = 1 mg/L), and, in combination with the mutations in *porB* and *mtrR*, contributes in the development of chromosomal resistance (MIC >2 mg/L) (15). High-level tetracycline resistance (MIC >16 mg/L) is plasmid-mediated and caused by the presence of the *tetM* determinant (3). Mutations in *gyrA* and *parC*, that encode DNA gyrase A and topoisomerase IV, respectively, are correlated with ciprofloxacin resistance (16). Resistance to macrolides, such as azithromycin, is caused by mutations in 23S r-RNA, coupled with mutations in *mtrR* (17, 18).

In Saskatchewan (SK), more than 95% of *N. gonorrhoeae* cases are diagnosed by NAAT using the Health Canada approved Aptima Combo 2 Assay (19, 20). Thus, there is no information on the AMS of these gonococcal specimens. Only two investigations have tested remnant Aptima specimens to predict AMR and strain types (STs) of *N. gonorrhoeae* using molecular methods (21, 22). Pabbaraju et al. purified DNA from 34 *N. gonorrhoeae* positive Aptima specimens, followed by PCR and DNA sequencing, to determine mutations in PBP2 (*penA*) linked with resistance to cefixime, and to ascertain STs using the *N. gonorrhoeae* multi-Antigen Sequence Typing (NG-MAST) method (21). Melendez et al. purified DNA from 552 remnant Aptima swabs positive for *N. gonorrhoeae* and used qPCR to predict resistance to ciprofloxacin (*gyrA* and *parC*), penicillin (*blaTEM* plasmid-mediated penicillin resistance), and ESCs – cefixime and ceftriaxone (*penA*) (22). The present research aimed to predict susceptibility based on the molecular characterization of AMR genes by utilizing remnant Aptima urine specimens. We isolated DNA from 50 *N. gonorrhoeae* positive remnant Aptima urine specimens to predict AMR to six antibiotics. We amplified and sequenced *penA*, *porB*, *mtrR*, *ponA*, *rpsJ*, *gyrA*, *parC*, and 23S r-RNA from these specimens to ascertain the mutations linked with different AMRs and characterized AMR STs using the *Neisseria gonorrhoeae* Sequence Typing for Antimicrobial Resistance (NG-STAR)

program (23). We also ascribed STs by NG-MAST, which characterizes two hypervariable loci, *tbpB* and *porB* (24). These analyses allowed us to generate insights into the emerging resistance and STs of *N. gonorrhoeae* in SK that would otherwise not have been possible.

5.3 MATERIALS AND METHODS

5.3.1 Specimen Collection

N. gonorrhoeae was identified from urine specimens submitted to the Roy Romanow Provincial Laboratory (RRPL, Regina, SK) using the Aptima Combo 2 assay (Hologic Gene-Probe Inc., CA, USA). Information such as place and date of collection, age, and sex of the patients was also collected. Fifty *N. gonorrhoeae* positive remnant Aptima urine specimens (40 were collected in June/July 2017 and 10 in November 2017) were transported to the University of Saskatchewan on dry ice and stored at -20°C. Patient ages ranged from 15-59; 20 were male, 30 were female.

5.3.2 DNA Extraction & Amplification of AMR Genes from Remnant Aptima Urine Specimens

DNA from specimens collected during June/July 2017 (n=40) was isolated using the QIAamp DNA Mini kit (Qiagen, Ontario; method 1) as per the manufacturer's instructions. For method 1, *Taq* polymerase was used to amplify (55°C annealing temperature) eight (*penA*, *porB*, *mtrR*, *ponA*, *gyrA*, *parC*, *rpsJ* and 23S r-RNA) AMR determinants and two NG-MAST loci (*tbpB* and *porB*). However, due to difficulties in the amplification of AMR determinants in many specimens from method 1, DNA from all 50 specimens was purified using the QIAamp Viral RNA Mini kit (Qiagen, Ontario; method 2). For method 2, 400µl of the Aptima specimen was spun at 3000g for 1 min and the supernatant was processed using the manufacturer's instructions. Phusion High fidelity DNA polymerase (New England Biolabs, NEB) was used to amplify various genes (that were not previously amplified) from the DNA obtained using method 2. Here, AMR determinants were amplified using the following annealing temperatures: penA-A1/penA-B1 62^{o}C; penA-A2/penA-B2 55^{o}C; penA-A3/penA-B3 58^{o}C; mtrR1/mtrR2 58^{o}C, por-NGMAST-F/por-NGMAST-R 58^{o}C; ponA1-F/ponA1-R 65^{o}C, GYRA-1/GYRA-2 68^{o}C; parC-F/parC-R 62^{o}C; gonrRNAF/gonrRNAR2 55^{o}C; tbpB-F/tbpB-R 69^{o}C; and rpsJ-F/rpsJ-R 55^{o}C.

For both methods, DNA concentrations were measured by using NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Ontario) and 260/230 and 260/280 ratios were recorded. Primer sequences (Table 5.1) for *rpsJ* (25) and other AMR determinants (https://ngstar.canada.ca/) were synthesized at Thermo Fisher Scientific. Amplicons were

confirmed for the correct size (https://ngstar.canada.ca/) by agarose gel electrophoresis and were sequenced by Sanger sequencing (Eurofins Genomics, Ontario). Amplicon products with distinct bands were directly submitted for sequencing. Only amplicons containing primer-dimers or nonspecific amplification were purified, using the QIAquick PCR Purification Kit (Qiagen), and subsequently sequenced. Differences in DNA concentration for each specimen and 260/230 ratios obtained by the two methods were evaluated using the paired t-test in GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA).

5.3.3 DNA Sequence Analysis & Strain Typing

penA sequences were assembled using Clone Manager 9 Professional (Scientific & Educational Software, NC, USA). Nucleotide sequences of all genes (except for 23S r-RNA) were translated into protein sequences and mutations implicated in AMR were identified by manually aligning to the reference sequences downloaded from NG-STAR (https://ngstar.canada.ca/). The reference sequence for *rpsJ* was retrieved from NCBI (*N. gonorrhoeae* FA19 - GenBank: EEZ46595.1). The manual alignment of each nucleotide and protein sequence to reference sequences was carried out using CLC Genomics Workbench (Qiagen). The allele numbers for each AMR determinant were obtained and NG-STAR STs were assigned. NG-MAST STs were obtained by aligning DNA sequences to the ST database at the NG-MAST website (www.ng-mast.net/). The phylogenetic tree was constructed using the maximum likelihood method in MEGA7 after concatenating each AMR determinant.

5.3.4 Prediction of AMR

AMR was predicted according to criteria reported in the literature. Reduced susceptibility to cefixime (MIC ≥0.25 mg/L) can be associated with non-mosaic PBP2 (*penA*) types such as 13.001 (A501V substitution) or mosaic PBP2 (penA) types such as 10.001 and 34.001 with associated I312M, V316T, G545S, A501V, and N512Y substitution (12, 14, 26, 27). In addition, isolates with reduced susceptibility to cefixime also carry mutations in MtrR (*mtrR*, e.g. A-; A38T; G45D) and PIB (*porB*, e.g. G120K; A121N/S/G; 12).

Mosaic PBP2 (*penA*) types coupled with the substitution G542S and P551S/I are associated with elevated MICs (MIC ≥0.016 mg/L) to ceftriaxone (28). Also, substitution such as A311V and

Table 5.1 List of primers used to amplify *N. gonorrhoeae* AMR markers.

AMR marker	Primer name	Primer sequence (5' to 3')	Product size (bp)	Reference
penA	PenA-A1(forward) PenA-B1(reverse) PenA-A2(forward) PenA-B2 (reverse) PenA-A3(forward) PenA-B3 (reverse)	CGGGCAATACCTTTATGGTGGAAC AACCTTCCTGACCTTTGCCGTC AAAACGCCATTACCCGATGGG TAATGCCGCGCACATCCAAAG GCCGTAACCGATATGATCGA CGTTGATACTCGGATTAAGACG	669 581 863	23
mtrR	MTR1(forward) MTR2 (reverse)	AACAGGCATTCTTATTTCAG TTAGAAGAATGCTTTGTGTC	916	23
porB1b	por-NGMAST-F (forward) por-NGMAST-R (reverse)	CAAGAAGACCTCGGCAA CCGACAACCACTTGGT	737	23
ponA	ponA1-f (forward) ponA1-r (reverse)	CGCGGTGCGGAAAACTATATCGAT AGCCCGGATCGGTTACCATACGTT	1240	23
gyrA	GYRA-1(forward) GYRA-2 (reverse)	AACCCTGCCCGTCAGCCTTGA GGACGAGCCGTTGACGAGCAG	270	23
parC	parC F (forward) parC R (reverse)	GTTTCAGACGGCCAAAAGCC GGCATAAAATCCACCGTCCCC	332	23
23S rRNA	gonrRNAF (forward) gonrRNAR2 (reverse)	ACGAATGGCGTAACGATGGCCACA TTCGTCCACTCCGGTCCTCTCGTA	712	23
rpsJ	RPS-for RPS-rev	GTGCTGTTGTAAAAGGCCCG CGGCCGGCAAATCCAGCTTC	186	25

T483S in mosaic PBP2 type 60 confer reduced susceptibility to ceftriaxone (29). Some reports suggest that MtrR (*mtrR*), PIB (*porB*) and other novel genes are implicated in reduced susceptibility to ceftriaxone (MIC ≥0.125 mg/L) (27, 30, 31).

Resistance to azithromycin is conferred by A2059G (high level) and C2611T (low level) in all four alleles of 23S r-RNA (32). Mutations in MtrR (i.e. A-; A38T; G45D) are also implicated in high-level resistance of *N. gonorrhoeae* to azithromycin (18). Several substitutions at positions S91 (e.g. S91F) and D95 (e.g. D95G/A/N) of GyrA (*gyrA*), and positions D86, S87 and S88 of ParC (*parC*, e.g. D86N; S87R/N; S88P; and E91K) are associated with resistance to ciprofloxacin (MIC ≥1 mg/L) (33-35). The step-wise acquisition of mutations in *penA, mtrR, porB* and *ponA* confers stepwise increases in MIC to penicillin (10, 36). Insertion of aspartic acid at position 345 in PBP2, together with an additional four and/or eight substitutions (e.g. F504L, A510V, A516G, H541N and/or P552V, K555Q, I556V, and I557V) in any non-mosaic PBP2 (*penA*) confer an intermediate MIC to penicillin (MIC = 0.12 mg/L) (10). Mutations (i.e. A-; A38T; G45D) in MtrR (*mtrR*) do not elevate penicillin MICs alone but in combination with G120K/N and A121N/S/G substitutions in PIB (*porB*) are associated with a penicillin MIC of 1 mg/L (10). The further addition of substitution L421P in PBP1 (*ponA*) confers resistance to penicillin (MIC ≥2 mg/L; 10).

For tetracycline, mutations in MtrR (*mtrR*, A-; A38T; G45D), PIB (*porB*, G120K:A121N; A121S; G120N:A121G) and S10 (*rpsJ*, V57M) can produce chromosomal resistance (MIC ≥ 2 mg/L) (15). The V57M substitution in S10 (*rpsJ*) must be present as mutations in *mtrR* or *mtrR*/*porB* do not confer high-level chromosomally-mediated resistance to tetracycline (15).

5.4 RESULTS

5.4.1 Assessment of DNA Extraction Methods for Amplification

DNA concentrations and 260/230 ratios were compared from the DNA of 40 Aptima urine specimens extracted using methods 1 and 2. Specimens processed with the Viral RNA Mini kit (method 2) exhibited significantly ($P < 0.01$, paired t-test; Figure 5.1) higher DNA concentrations (96.21±2.683 ng/µl) as compared with specimens processed using the DNA Mini kit (30.58±3.919 ng/µl; method 1). It should be noted that some carryover absorption of RNA using the Viral RNA Mini kit might have increased the apparent DNA concentration. We also observed that the 260/280 ratio was higher than 2 in >60% of the specimens processed with the Viral RNA Mini kit, which was probably caused by the carrier RNA added as recommended by the manufacturer. Furthermore, 85% (34/40) of the specimens processed by method 2 had better 260/230 ratios (1.9±0.11) as compared to specimens prepared using method 1 (0.58±0.02; $P < 0.01$, paired t-test; Figure 5.1).

If 100% amplification of the 8 AMR genes for each of the 50 specimens was achieved, 400 amplicons would have been generated. We obtained 397 amplicons when both methods 1 and 2 were combined (3 amplicons were not obtained due to insufficient specimen volumes). With method 1, only 146 of 320 (45.6%) amplicons were generated and 251 amplicons of 397 (63.2%) were produced using method 2. With method 1, the sequences of 97.2% (142/146) of the amplicons were characterized (i.e. defined allele numbers upon complete alignment with the reference NG-STAR database). With method 2, 91.2% (229/251) of the DNA sequences were assigned to NG-STAR alleles (data not shown).

5.4.2 Mutations Observed in 8 AMR Genes of *N. gonorrhoeae* Positive Aptima Urine Specimens

Overall, the sequences of AMR determinants PBP1, GyrA, ParC and 23S r-RNA, were characterized for 96, 98, 100 and 100% of the specimens, respectively. The sequences of PBP2, PIB, MtrR, and S10 were characterized for 94, 90, 82, and 86% of the specimens, respectively. Sequence analysis of PBP2 (*penA*) revealed that 47/50 specimens which were characterized, carried 2 mosaic (34.001 and 67.001), 2 semi-mosaic (91 and 80) and 6 non-mosaic

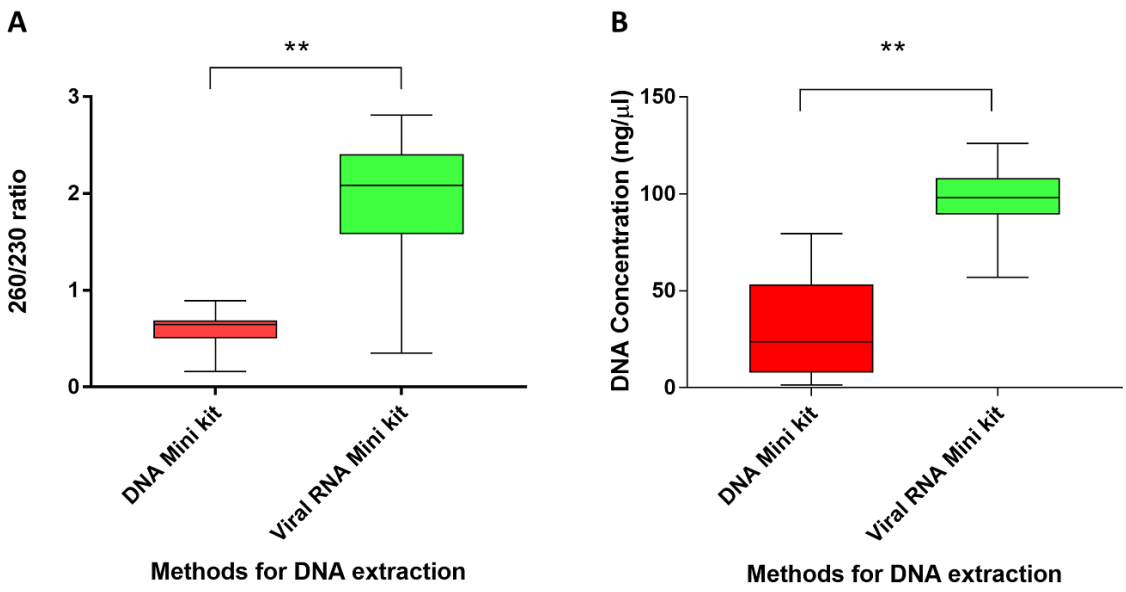


Figure 5.1 DNA purification from remnant Aptima urine specimens using DNA Mini kit and Viral RNA Mini kit. **A)** Comparison of 260/230 ratios of DNA. X-axis: Name of kit; Y-axis: 260/230 ratio. **B)** Comparison of DNA concentrations. X-axis: Name of extraction kit; Y-axis: Concentration of DNA (ng/µl).

(1.001, 2.001, 12.001, 14.001/14.002, 22.001 and 61.001; Table 5.2) PBP2 types. Mosaic PBP2 (*penA*) patterns 34.001 (20%, 10/50) and 67.001 (4%, 2/50) comprised 24% of the specimens, and semi-mosaic PBP2 patterns 91.001 (8%, 4/50) and 80 (2%, 1/50) comprised 10% of the specimens. Non-mosaic PBP2 patterns 14.001/14.002 (40%, 20/50), 12.001 (12%, 6/50), 2.001 (2%, 1/50), 22.001 (2%, 1/50), 61.001 (2%, 1/50) and 1.001 (2%, 1/50) comprised 60% (30/50) of the specimens. PBP2 sequences in 3 specimens could not be characterized due to their poor quality.

A high percentage (18%, 9/50) of the specimens could not be characterized for mutations in MtrR (*mtrR*) due to the poor quality of the sequences. 28% (14/50) carried wild-type sequences of the MtrR (*mtrR*) repressor and upstream region, while 24% (12/50) carried an A- deletion in the promoter region, and 20% (10/50) carried an A39T substitution in MtrR (*mtrR*) (Table 5.2). Mutation combinations such as A-; A39T (10%, 5/50) and A-: G45D (2%, 1/50) were also noted.

For PIB (*porB*), 90% of the alleles could be characterized (45/50) (Table 5.2). Wild-type sequences of PIB (*porB*) comprised 22% (11/50) of the specimens. Substitutions such as G120K:A121N (34%, 17/50), A121S (20%, 10/50) and G120N:A121G (6%, 3/50) were also identified in PIB (Table 5.2). *porB1a* alleles were observed in 10% (5/50) of the specimens. Wild-type sequences of PBP1 were observed in 66% (33/50) of the specimens and an L421P substitution was noted in 30% (15/50) (Table 5.2). PBP1 (*ponA*) sequences in 4% (2/50) of the specimens could not be characterized. Only 8% (4/50) of S10 (*rpsJ*) sequences were wild-type, 78% (39/50) carried a V57M substitution (Table 5.2). S10 (*rpsJ*) sequences in 8% (4/50) could not be characterized, and 6% (3/50) could not be tested due to insufficient specimen volume.

All but one of the GyrA (*gyrA*, 49/50) and all ParC (*parC*, 50/50) sequences were ascertained (Table 5.2). High percentages of wild-type DNA sequences of *gyrA* (74%, 37/50) and *parC* (74%, 37/50) were observed. Some specimens carried GyrA substitutions S91F;D95G (24%, 12/50) and S87R (26%, 13/50) in ParC. Since substitutions in GyrA are predictive of resistance to ciprofloxacin, these results predict that three-quarters of the specimens were susceptible to this antibiotic. All specimens could be characterized for potential substitutions in the peptidyl-

Table 5.2 Prevalent mutation patterns of chromosomal AMR determinants identified from remnant Aptima urine specimens.

AMR markers (n = 50)	Allele type	Pattern/Mutations	n (%)
PBP2 *(penA)* **n_{penA} =47**	Non-mosaic	14.001/14.002	20 (40)
		12.001	6 (12)
		2.001	1 (2)
		22.001	1 (2)
		61.001	1 (2)
		1.001	1 (2)
	Mosaic	34.001	10 (20)
		67.001	2 (4)
	Semi-mosaic	91.001	4 (8)
		80.001	1 (2)
	Unable to characterize	-	3 (6)
MtrR (*mtrR*) **n_{mtrR} = 41**	Wild type	WT	14 (28)
	Mutated	A-	12 (24)
		A39T	10 (20)
		A-, A39T	5 (10)
		A-, G45D	1 (2)
	Unable to characterize	-	9 (18)
PIB (*porB*) **n_{porB} = 45**	Wild type	WT	11 (22)
		porB1a	5 (10)
	Mutated	G120K; A121N	17 (34)
		A121S	10 (20)
		G120N; A121G	3 (6)
	Unable to characterize	-	5 (10)
PBP1 (*ponA*) **n_{ponA}= 48**	Wild type	WT	33 (66)
	Mutated	L421P	15 (30)
	Unable to characterize	-	2 (4)
S10 (*rpsJ*) **n_{rpsJ}= 43**	Wild type	WT	4 (8)
	Mutated	V57M	39 (78)
	Unable to characterize	-	7 (14)
GyrA (*gyrA*) **n_{gyrA}= 49**	Wild type	WT	37 (74)
	Mutated	S91F; D95G	12 (24)
	Unable to characterize	-	1 (2)
ParC (*parC*) **n_{parC} = 50**	Wild type	WT	37 (74)
	Mutated	S87R	13 (26)
	Unable to characterize	-	-
23S r-RNA **$n_{23S\ rRNA}$= 50**	Wild type	WT	47 (94)
	Mutated	A2059G	3 (6)
	Unable to characterize	-	-

transferase loop region in domain V of 23S r-RNA; 94% (n=47/50) of the specimens carried a wild-type 23S r-RNA whereas 6% (n=3/50) had A2059G substitution in 23S r-RNA (Table 5.2). Therefore, these results indicate that 94% of the specimens could be predicted as being susceptible to azithromycin. As only one allele was sequenced, the identification of 3 specimens as being resistant is presumptive.

5.4.3 Prediction of AMR in *N. gonorrhoeae* Remnant Aptima Specimens

The 8 AMR determinants tested could be characterized completely in 36/50 specimens (Table 5.3, Figure 5.2). As the characterization of multiple genes (*penA*, *mtrR*, *porB*, *rpsJ*, *ponA*) is required to predict susceptibility to cefixime, penicillin, and tetracycline, the denominator used for these antibiotics was 36. However, the denominator used to predict ciprofloxacin and azithromycin resistance was 50, reflecting the high number of relevant genes characterized. A phylogenetic tree, constructed by concatenating the sequences of these genes (Figure 5.2), showed that specimens were clustered based on similar NG-STAR profiles. Two major clusters were observed. Cluster-A was distinguished by semi-mosaic (cluster A1) and mosaic (cluster A2) PBP2 patterns. Cluster-B comprised non-mosaic PBP2 types with Cluster B1 specifying PBP2 types 14.001/14.002 and 22.001 and Cluster B2 specifying PBP2 types 2.001, 12.001, and 61.001.

The 3 specimens in cluster A1 carried a novel PBP2 pattern 91.001, identified in this research (similar to PBP2 pattern 39.001 with additional substitutions N512Y, G545S, T550A, and V566I; Figure 5.2; sequence available at https://ngstar.canada.ca/). Substitutions such as N512Y/G545S, in association with I312M, V316T, are implicated in elevated MICs to cefixime (12). However, specimens in Cluster A1 did not carry I312M, V316T substitutions and hence these specimens were predicted to be susceptible to cefixime (Figure 5.2). Specimens in cluster A2 carried mosaic PBP2 types 67.001 (n=1) and 34.001 (n=10; Figure 5.2). These specimens (11/36, 30.5%) carried PBP2 substitutions (I312M, V316T, N512Y, and G545S) implicated in reduced susceptibility to cefixime. None of the Cluster B specimens, which comprised non-mosaic PBP2 types (1.001, 2.001, 12.001, 14.001, 22.001 and 61.001; Figure 5.2), was predicted to be resistant to cefixime. Overall, 30.5% (11/36) of the specimens were predicted to possibly have reduced susceptibility to cefixime. None of the specimens was predicted to have reduced susceptibility to ceftriaxone since

the documented substitutional combinations such as A311V;T483S (in PBP2 pattern 60.001) associated with reduced susceptibility (MIC ≤ 0.125 mg/L) and G542S;P551L/S (in any PBP2 patterns) associated with elevated MICs to ceftriaxone (MIC ≤ 0.016 mg/L) were not observed in this research. However, 3 specimens in cluster B2 (nos. 13, 17, and 9) with PBP2 pattern 12.001 carried a P551S substitution (Figure 5.2). These specimens were considered to be susceptible to ceftriaxone as an additional G542S substitution is associated with elevated MICs (MIC ≥0.016 mg/L) to ceftriaxone (28).

In Cluster A, two specimens (nos. 42 and 45) carried the substitution A2059G in 23S r-RNA predictive of resistance to azithromycin (Figure 5.2). One specimen from Cluster B (no. 47) also carried an A2059G substitution in 23S r-RNA predictive of high-level resistance to azithromycin.

Eleven specimens in Cluster A carried substitutions in both GyrA and ParC and were predicted to be resistant to ciprofloxacin. None of the specimens in cluster B had substitutions in GyrA or ParC (Figure 5.2). However, one additional specimen, which was partially characterized, was also predicted to be resistant to ciprofloxacin (Table 5.3). In total, 24% (12/50) of the specimens were predicted to be ciprofloxacin-resistant.

In Cluster A, eight of 11 specimens (nos. 24, 42, 45, 2, 29, 34, 18, and 36) had mutations PBP2, MtrR, PIB and PBP1 (PBP2/MtrR/PIB/PBP1), which are associated with the sequential acquisition of chromosomal resistance to penicillin. In Cluster B, two specimens (nos. 9 and 10) had mutations in PBP2/MtrR/PIB/PBP1 predictive of resistance to penicillin (Figure 5.2). A total of 90.9% (20/22) of the specimens did not have mutations in all AMR determinants (PBP2/MtrR/PIB/PBP1), which are associated with the development of resistance to penicillin and hence these specimens were predicted to be susceptible or have intermediate MICs (<2 mg/L) to penicillin. Overall, 27.7% (10/36) of the specimens were predicted as being penicillin-resistant. Nine specimens (i.e. nos. 38, 24, 31, 42, 45, 2, 29, 18, and 36) in Cluster A carried mutations in MtrR, PIB and S10 predictive of chromosomally mediated resistance to tetracycline (Figure 5.2). In Cluster B, six specimens (nos. 23, 47, 27, 50, 9 and 10) carried such mutations (Figure 5.2). Overall, 41.6% (15/36) of the fully characterized specimens were predicted as being tetracycline resistant.

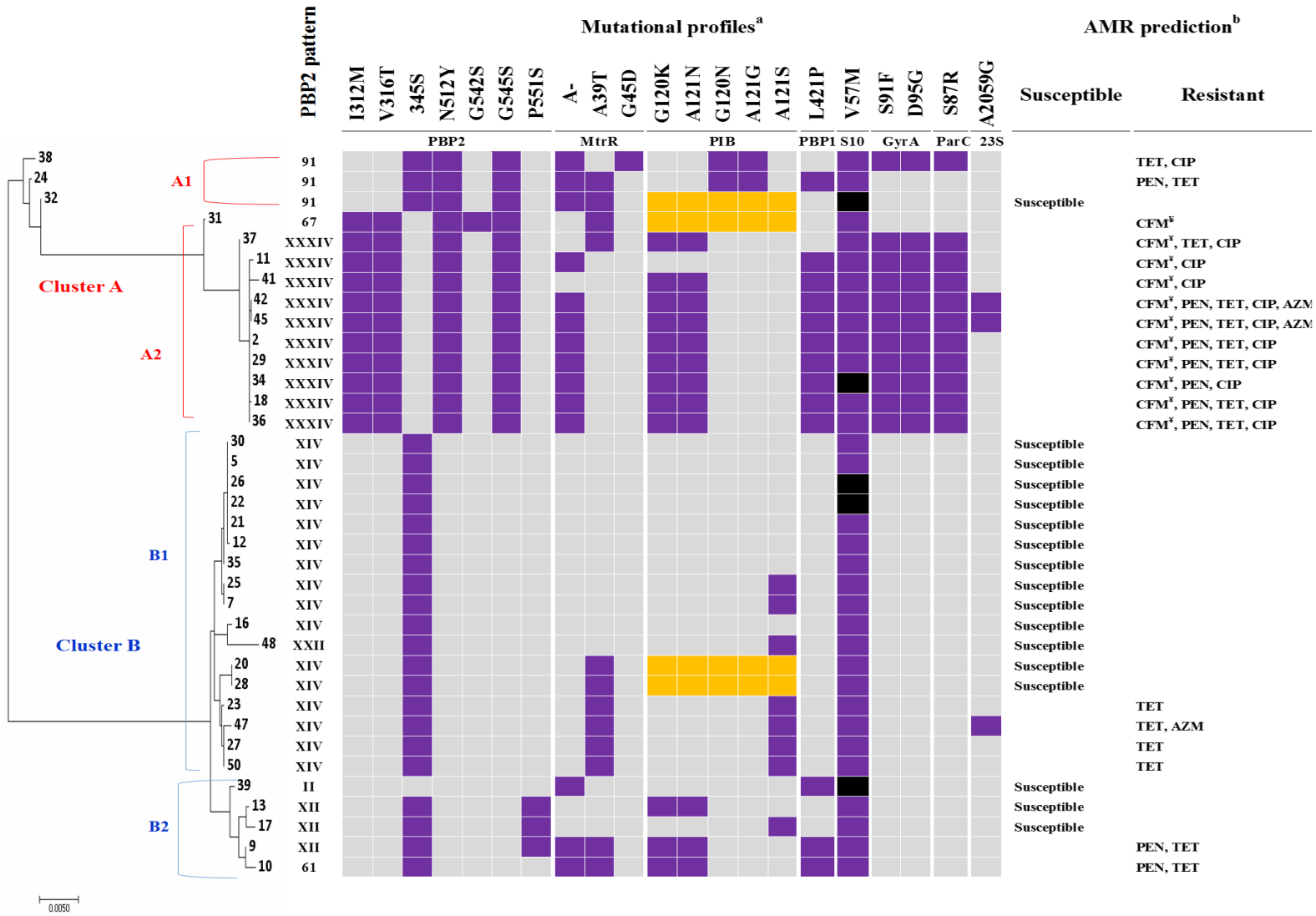


Figure 5.2 Phylogenetic tree of the Aptima urine specimens clustering based on their allelic profiles of 8 AMR determinants; AMR prediction based on the association of mutations with respective antibiotics. Columns indicate the mutation patterns for each molecular determinant. ¥ indicates possibly decreased susceptibility to cefixime. The complete profile of 14/50 samples could not be ascertained.

Table 5.3 Mutations in chromosomal AMR determinants, NG-STAR and NG-MAST STs from 50 remnant Aptima urine specimens.

Specimen no, %	**Penicillin**					**Ciprofloxacin**		**Azithromycin**	**Strain Types (STs)**	
(n=50)	**ESCs**		**Tetracycline**						**NG-STAR**	**NG-MAST (n)**
	penA*	***ponA***	***mtrR***	***porB***	***rpsJ (n)***	***gyrA***	***parC***	**23S r-RNA**		
8 (16%)	14.002[a]	WT[d]	WT	WT	V57M(6), - (2)	WT	WT	WT	160	7638 (5), 11933 (1), 14878 (1), 14537 (1)
5 (10%)	34.001[c]	L421P	A-	G120K; A121N	V57M(4), na (1)	S91F; D95G	S87R	WT	90	10451 (1), 10956 (1), 16196 (1), 11299 (1), - (1)
2 (4%)	34.001[c]	L421P	A-	G120K; A121N	V57M	S91F; D95G	S87R	A2059G	1018†	10451 (2)
2 (4%)	14.001[a]	WT	WT	A121S	V57M	WT	WT	WT	191	800 (1), - (1)
2 (4%)	14.001	WT	A39T	*porB1a*	V57M	WT	WT	WT	289	-
2 (4%)	14.001	WT	A39T	A121S	V57M	WT	WT	WT	42	6968 (1), 5985 (1)
1 (2%)	34.001	L421P	WT	G120K; A121N	V57M	S91F; D95G	S87R	WT	88	4951
1 (2%)	34.001	L421P	A-	WT	V57M	S91F; D95G	S87R	WT	89	11691
1 (2%)	2.001[a]	L421P	A-	WT	-	WT	WT	WT	442	5441
1 (2%)	14.001	WT	A39T	A121S	V57M	WT	WT	WT	1021†	5985
1 (2%)	14.001	WT	A39T	A121S	V57M	WT	WT	A2059G	1017†	5985
1 (2%)	22.001[a]	WT	WT	A121S	V57M	WT	WT	WT	1016†	5985
1 (2%)	12.001[a]	L421P	A-; A39T	G120K; A121N	V57M	WT	WT	WT	1024†	3912
1 (2%)	61.001[a]	L421P	A-; A39T	G120K; A121N	V57M	WT	WT	WT	1023†	3912
1 (2%)	12.001	WT	WT	G120K; A121N	V57M	WT	WT	WT	1022†	17008‡
1 (2%)	91.001[b†]	L421P	A-; A39T	G120N; A121G	V57M	WT	WT	WT	1398†	17005‡
1 (2%)	34.001	WT	A39T	G120K; A121N	V57M	S91F; D95G	S87R	WT	1019†	17006‡
1 (2%)	91.001	WT	A-; A39T	*porB1a*	V57M	WT	WT	WT	1397†	-
1 (2%)	91.001	WT	A-;G45D	G120N; A121G	V57M	S91F; D95G	S87R	WT	1396†	-

1 (2%)	12.001	WT	WT	A121S	V57M	WT	WT	WT	1025†	-
1 (2%)	67.001[c]	WT	A39T	*porB1a*	V57M	WT	WT	WT	1020†	-
1 (2%)	12.001	-	-	G120K; A121N	-	WT	WT	WT	-	3912
1 (2%)	12.001	WT	-	G120K; A121N	WT	WT	WT	WT	-	-
1 (2%)	12.001	WT	-	G120K; A121N	WT	WT	WT	WT	-	-
1 (2%)	14.002	WT	-	G120K; A121N	V57M	WT	WT	WT	-	-
1 (2%)	14.002	WT	-	WT	WT	WT	WT	WT	-	-
1 (2%)	-	L421P	A-	G120K; A121N	V57M	S91F; D95G	S87R	WT	-	-
1 (2%)	67.001	L421P	A-	-	WT	WT	WT	WT	-	-
1 (2%)	1.001[a]	WT	A-	-	WT	WT	WT	WT	-	-
1 (2%)	14.002	WT	-	-	V57M	-	WT	WT	-	-
1 (2%)	-	WT	A39T	-	V57M	WT	WT	WT	-	-
1 (2%)	80.001[b]	WT	-	A121S	V57M	WT	WT	A2059G	-	-
1 (2%)	14.002	WT	-	-	V57M	WT	WT	WT	-	-
1 (2%)	91.001	WT	-	*porB1a*	na	WT	WT	WT	-	-
1 (2%)	-	-	A39T	A121S	na	WT	S87R	WT	-	-

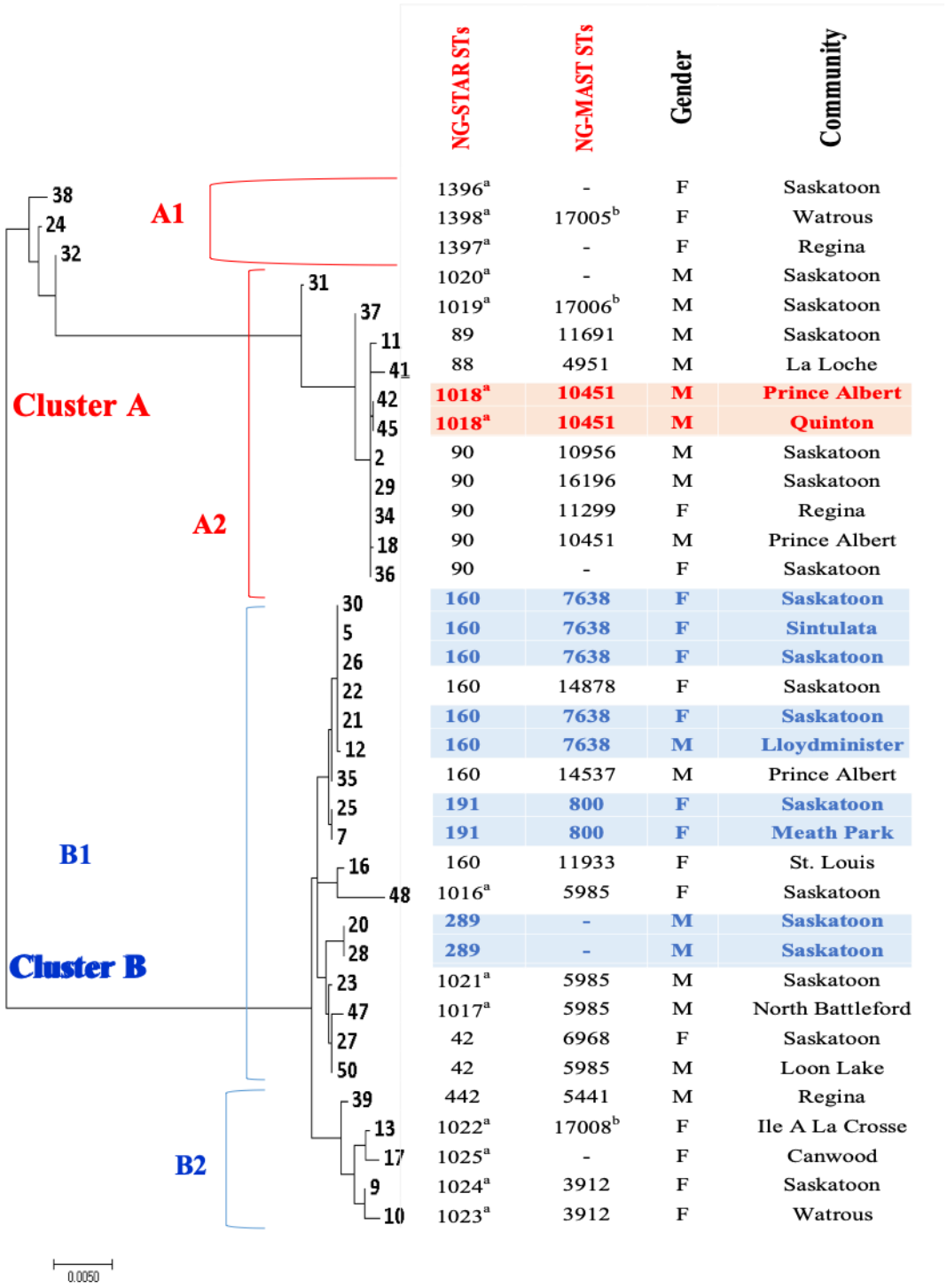
[*]*penA* types (NG-STAR numbers): [a]non-mosaic, [b]semi-mosaic and [c]mosaic
[d]Wild type allele
[†]New *penA* and new STs for NG-STAR
[‡]New ST for NG-MAST
[-]uncharacterized alleles and STs (NG-MAST and NG-STAR)
[na]specimens were not amplified due to insufficient volume

5.4.4 NG-MAST & NG-STAR STs & Demographics

NG-STAR STs could be assigned to 72% (n=36/50) of the specimens that were fully characterized for all AMR determinants (Table 5.3). A total of 21 different NG-STAR STs were assigned to 36 specimens (Table 5.3). NG-STAR STs such as 160 (22.2%; 8/36), 90 (13.8%; 5/36), 191 (5.5%; 2/36), 289 (5.5%; 2/36), and 42 (5.5%; 2/36) were predominantly observed (Table 5.3). We also observed new allelic combinations in 13/36 specimens and they were assigned to STs such as 1018, 1021, 1017, 1016, 1024, 1023, 1022, 1398, 1019, 1397, 1396, 1099, and 1020 (Table 5.3).

Successful strain typing by NG-MAST was achieved for 66% (n=33/50) of the specimens (Table 5.3, Figure 5.3), which comprised 19 different NG-MAST STs (Table 5.3). NG-MAST STs such as 7638 (15.1%; 5/33), 5985 (12.1%; 4/33), 3912 (9.09%; 3/33), and 10451 (9.09%, 3/33) predominated (Table 5.3). Three of 33 specimens had new allelic combinations of *porB* and *tbpB* (i.e. NG-MAST STs ST-17005, ST-17006, and ST-17008; Table 5.3).

There was mostly no association between NG-STAR and NG-MAST STs. However, 2 specimens (Figure 5.3, cluster A2) with NG-MAST ST-10451 were also NG-STAR ST-1018. Both specimens were isolated from males but from different geographic areas (Figure 5.3). In cluster B1, 5 specimens (4 female and 1 male) that were NG-STAR ST-160, were also NG-MAST ST-7638 (Figure 5.3). Two specimens with NG-STAR ST-191 also carried NG-MAST ST-800 and were collected from different regions. These limited data indicate potentially linked isolates.



NG-STAR STs	NG-MAST STs	Gender	Community
1396[a]	-	F	Saskatoon
1398[a]	17005[b]	F	Watrous
1397[a]	-	F	Regina
1020[a]	-	M	Saskatoon
1019[a]	17006[b]	M	Saskatoon
89	11691	M	Saskatoon
88	4951	M	La Loche
1018[a]	**10451**	**M**	**Prince Albert**
1018[a]	**10451**	**M**	**Quinton**
90	10956	M	Saskatoon
90	16196	M	Saskatoon
90	11299	F	Regina
90	10451	M	Prince Albert
90	-	F	Saskatoon
160	**7638**	**F**	**Saskatoon**
160	**7638**	**F**	**Sintulata**
160	**7638**	**F**	**Saskatoon**
160	14878	F	Saskatoon
160	**7638**	**F**	**Saskatoon**
160	**7638**	**M**	**Lloydminister**
160	14537	M	Prince Albert
191	**800**	**F**	**Saskatoon**
191	**800**	**F**	**Meath Park**
160	11933	F	St. Louis
1016[a]	5985	F	Saskatoon
289	**-**	**M**	**Saskatoon**
289	**-**	**M**	**Saskatoon**
1021[a]	5985	M	Saskatoon
1017[a]	5985	M	North Battleford
42	6968	F	Saskatoon
42	5985	M	Loon Lake
442	5441	M	Regina
1022[a]	17008[b]	F	Ile A La Crosse
1025[a]	-	F	Canwood
1024[a]	3912	F	Saskatoon
1023[a]	3912	F	Watrous

Figure 5.3 Phylogenetic tree of the Aptima urine specimens describing the strain types and their demographic details. New Allelic Combinations (NAC) for NG-STAR and NG-MAST STs are indicated with "a" and "b", respectively. Dash "-" indicates uncharacterized STs. Common NG-MAST and NG-STAR STs are coloured red in cluster A and blue in cluster B.

5.5 DISCUSSION

The extensive use of NAATs for the identification of *N. gonorrhoeae* from clinical specimens has significantly reduced the availability of cultured isolates for AMS testing. Thus, there is an urgent need for a validated molecular approach for the characterization of AMR determinants in *N. gonorrhoeae* positive NAAT specimens to predict AMS and/or AMR (37). In the present research, we used PCR and DNA sequencing approaches to characterize eight different genes implicated in gonococcal AMR from *N. gonorrhoeae* positive remnant Aptima urine specimens to predict the AMS.

The present research describes emerging resistance in *N. gonorrhoeae* from SK to antibiotics such as cefixime and azithromycin. Mosaic PBP2 type 34.001, which carries key substitutions such as I312M, V316T, N512Y, and G545S is associated with reduced susceptibility to cefixime (38). We predicted 30.5% (11/36) of the specimens would carry reduced susceptibility to cefixime due to these substitutions. Although the MIC cut-off for cefixime reduced susceptibility is 0.25 mg/L (27), the gonococcal isolates with PBP2 type 34.001 may have a range of MICs (MIC = 0.06 – 0.250 mg/L) to cefixime (39). Treatment failure to cefixime with the MIC of 0.125 mg/L has also been reported (40). Thus, our predictions are cautionary and imply a need for further surveillance of emerging resistance. A previous report from SK indicated that only 1 of 146 cultured isolates carried a mosaic *penA* allele with PBP2 type 34.001, and had a MIC of 0.125 mg/L for cefixime (41). PBP2 type 34.001 was first reported in the USA (42) and has spread worldwide (43, 44). In Canada, a study of gonococcal isolates (n=169) with reduced susceptibility to ESCs reported an increase in isolates with PBP2 type 34.001 from 2005 (25%, n=2/8) to 2010 (33%, n=14/43), with the highest prevalence in 2012 (50%, n=12/24) (45). We also determined, as in other studies, that gonococcal specimens with PBP2 type 34.001 were also resistant to ciprofloxacin due to predictive substitutions in GyrA and ParC (23, 46).

In the present research, none of the *N. gonorrhoeae* positive Aptima urine specimens was predicted to carry mutations implicated in reduced susceptibility to ceftriaxone. However, in Canada, a recent report showed two travel-related cases of ceftriaxone-resistant strains of *N. gonorrhoeae* which were genetically linked with *N. gonorrhoeae* strain FC428 identified in

Japan (2015) (47). In the NG-STAR database, PBP2 type 60.001 is associated with ceftriaxone resistance and carries A311V and T483S substitutions.

Three specimens (6%; 3/50) carried an A2059G substitution in the 23S r-RNA gene predictive of the development of high-level resistance to azithromycin. Although the substitution was observed in a single allele of 23S r-RNA, isolates with a substitution in just one allele are capable of rapidly acquiring the substitutions in all four alleles (48). Increasing rates of azithromycin resistance have been reported nationally (49) and globally in *N. gonorrhoeae* (50). In Canada, between 2010 and 2016, the proportion of azithromycin-resistant *N. gonorrhoeae* increased from 1.3% to 7.2% (49). The WHO indicated that 17 of 42 countries reported (2012-2013) azithromycin resistance in 5% or more of isolates (50). Such a high rate of azithromycin resistance is of concern as it is recommended for combination therapy with ceftriaxone.

In the present research, we predicted that 27.7% (10/36) of the specimens would be penicillin-resistant. In Canada, 6% of *N. gonorrhoeae* isolates were reported as being resistant to penicillin in 2004 and this increased to a high of 25.1% in 2010 (it was 18.2% in 2014) (51). In SK, penicillin resistance from 2006 to 2012, and in 2015, was observed in <5% of the population (a cut-off determined by WHO, above which an antibiotic should not be used for treatment). Reported penicillin resistance in SK between 2013 and 2014 was 27.5% and 13.5% respectively (20).

This research predicted that 41.6% (15/36) of the specimens were tetracycline resistant. The Public Health Agency Canada (PHAC) reported that between 2013 and 2015, tetracycline resistance in Canada, increased from 33% to 56.4% due to increases in both plasmid-mediated and chromosomal tetracycline resistance (51). Similarly, between 2003 and 2015, 11.8 to 89.1% of gonococcal isolates from SK were tetracycline resistant (20). In the present research, we did not test for plasmid-mediated tetracycline resistance (*tetM*), which is important in the determination of an accurate rate for overall tetracycline resistance in SK. In SK, 16.8% and 17.5% of *N. gonorrhoeae* isolates carried plasmid-mediated tetracycline resistance in 2014 and 2015, respectively (20).

Several studies have reported the correlation of substitutions in GyrA with resistance to

ciprofloxacin (52-54). In our research, 74% (37/50) of the specimens did not have substitutions in GyrA and ParC and were identified as susceptible. A higher number of specimens showing ciprofloxacin susceptibility also suggests that an effective, rapid molecular test predicting susceptibility could be used to direct individualized treatment, potentially sparing the use of ESCs (55). The implementation of such an approach has been reported in the United States, where the results obtained using GyrA diagnostic assay were 100% concordant with the susceptibility test results (53). The most prevalent NG-MAST ST in our research was ST-7638 (15.1%; 5/33). Previous NG-MAST typing of *N. gonorrhoeae* isolates (n= 85; 2016) in SK showed that STs 7638 (20%, 17/85), 11933 (13%, 11/85), 11299 (10%, 8/85), 5985 (8%, 7/85) and 10451 (7%, 6/85) were predominant (51). A recent report from Alberta also indicated that ST-7638 was a predominant ST comprising 12.7% (367/2891) of the gonococcal isolates screened, with a higher proportion observed in female patients (56). In the present research, four of five specimens with ST-7638 were from female patients.

Although we were able to predict resistance to several antibiotics from Aptima urine specimens using molecular methods, this research has several limitations. The method of DNA purification impacted the amplification of antibiotic resistance genes in the specimens. We determined that the Viral RNA Mini kit coupled with Phusion high fidelity DNA polymerase was a better method to amplify genes from these specimens. There might have been a carryover of RNA in the DNA extracted using the Viral RNA Mini Kit, and this increased the total nucleic acid concentration and 260/280 ratio. We did not consider this to be a major issue as targeted amplicon sequencing was performed and the Viral RNA Mini kit performed better for our purposes. The purpose of describing DNA methods in detail was to give an insight into some of the issues regarding DNA extraction from such specimens. There may be other approaches that are better and more cost-effective. Another limitation is that there were differences in the success with which different genes that could be fully characterized for mutations (i.e. *ponA, gyrA, parC,* and 23S r-RNA sequences were determined in more than 95% of the specimens, while *penA, mtrR, porB,* and *rpsJ* were characterized in 82% to 94% of the specimens). Other studies have reported lower detection of markers such as *penA, mtrR,* and *porB* in clinical specimens using qPCR assays (38, 57, 58). In one study, 18.8–31.2% of the NAAT positive clinical specimens (n=1629) remained uncharacterized for markers such as *mtrR, porB, gyrA, and* 23S r-RNA (58). A second study

noted false-positive results in detecting of the Asp345del in *penA* from pharyngeal specimens using a qPCR assay (57). Additionally, cross-reactivity of *penA*, *mtrR*, *porB*, and *ponA* with similar genes from commensal *Neisseria* isolates has been reported in PCR assays using clinical specimens (38).

Furthermore, *penC*, *ermA*, 16S r-RNA, *tetM*, bla_{TEM} and several other genes predictive of resistance to penicillin, azithromycin, and spectinomycin were not assayed in our research due to logistical and cost issues. Historically, SK has a low prevalence of penicillinase-producing (0.15%) or plasmid-mediated tetracycline resistant *Neisseria gonorrhoeae* isolates (5.8%), but it would have been useful to ascertain the percentage of isolates carrying *blaTEM* and *tetM* (20). It should be noted that the prediction of resistance, based on molecular profiles of AMR genes, does not always align with MICs indicative of resistance and therefore carry an implicit error factor. The approach used by us is useful to monitor trends in emerging resistance given that over 90% of cases of *N. gonorrhoeae* in the province are not tested for AMR. We wish to highlight two other factors. The present research analyzed only remnant Aptima urine specimens (predominant specimen type collected in the province). Moreover, the utilization of costlier and labour-intensive first-generation sequencing, such as Sanger sequencing, limits the application of this AMR prediction approach in routine diagnostics.

A better approach for gonococcal AMR prediction and strain relatedness determination in clinical specimens would be whole-genome sequencing (WGS) as it could potentially provide comprehensive information regarding AMR genes and STs and allow for more rapid selection of appropriate antimicrobial therapy. This approach has been used for AMS surveillance using cultured *N. gonorrhoeae* isolates (59-63). Although WGS from Aptima urine or other clinical specimens identified by NAATs would be rapid and of great advantage, the extraction of DNA from such specimens presents notable challenges. For example, Aptima buffer is a transportation buffer which lyses all cells (human and bacterial) in the specimen, making it very difficult to enrich *N. gonorrhoeae* and other bacterial DNA (64, 65, unpublished observations). A few studies have performed bacterial enrichment (*Chlamydia trachomatis* and other bacteria) for WGS from urine specimens (64, 66). Only one study reported the enrichment of *N. gonorrhoeae* from urine specimens (67).

5.6 CONCLUSION

The molecular approach used in this research for the prediction of AMS in *N. gonorrhoeae* positive remnant Aptima urine specimens has the potential to preclude the culture-based AMS testing. However, the recovery of the sequences of substandard quality for some of the markers from such specimens limits their use to predict AMR. Furthermore, such an approach is costly, time-consuming and requires considerable expertise. The WGS analysis of such specimens would provide the advantage of covering an array of genes to characterize AMR and *N. gonorrhoeae* strain relatedness simultaneously. The development of methods to enable the WGS approach from clinical specimens is urgently needed.

5.7 SUMMARY POINTS

- The DNA sequences of *ponA, gyrA, parC* and 23S r-RNA alleles were determined in more than 95% of the remnant Aptima urine specimens, while *penA, mtrR* and *porB* were characterized in 86–94% of the specimens.
- The emergence (30.5%, 11/36) of *N. gonorrhoeae* with PBP2 type 34.001, which is associated with reduced susceptibility to cefixime, was observed and these specimens were also resistant to ciprofloxacin. These results reflect the expansion of this clone in Canada.
- No mutations indicative of reduced susceptibility to ceftriaxone were identified.
- NG-MAST ST-7638 is a circulating strain in western Canada and is associated with NG-STAR ST-160.
- A total of 47.2% (17/36) of the specimens were predicted as being fully susceptible to all antibiotics indicating that a molecular test to predict susceptibility would enhance the stewardship of antibiotics. When the determinants specifying ciprofloxacin resistance alone are considered, 76% (38/50) of the isolates were susceptible.

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5.9 FINANCIAL & COMPETING INTERESTS DISCLOSURE

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5.10 ETHICAL CONDUCT OF RESEARCH

An approval for processing *N. gonorrhoeae* positive Aptima urine specimens was obtained from ethical committee members of the University of Saskatchewan (Ethics approval no. 16–228).

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CHAPTER 6: A β-LACTAMASE-PRODUCING PLASMID FROM *NEISSERIA GONORRHOEAE* CARRIES A UNIQUE 6 BP DELETION IN *BLA*$_{\text{TEM-1}}$ ENCODING A TRUNCATED 24 KDa TEM-1 PENICILLINASE THAT HYDROLYZES AMPICILLIN SLOWLY

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6.0 INTERFACE

As discussed in Chapter 5, mutations in both the chromosome and plasmids can lead to drug resistance in *N. gonorrhoeae*. In Chapter 5, we mainly focused on mutations in the chromosome leading to resistance. In this research, we characterized a β-lactamase producing plasmid from *N. gonorrhoeae* with a novel 6 bp deletion in the ATG start codon of *bla*$_{\text{TEM-1}}$. This highlights the importance of extended monitoring for β-lactamase activity, as slow β-lactamase producing gonococcal strains could be easily misdiagnosed as non-PPNG isolates. Findings of plasmids harbouring *bla*$_{\text{TEM}}$ variants, such as pJRD20, is of critical importance for the proper control and

management of antibiotic resistance in *N. gonorrhoeae*. This chapter was previously published in *The Journal of Antimicrobial Chemotherapy*, 74(10): 2904-2912, 2019, and is reprinted here with permission. The findings of this research have been presented at the International Union Against Sexually Transmitted Infections (IUSTI) World + European Congress held in Dublin, Ireland on June 27-30th, 2018, and at the Protein Structure, Function and Malfunction (PSFaM) 7th Annual Meeting held in Saskatoon, Saskatchewan, Canada on June 20-21th, 2019.

Reema Singh analyzed DNA sequences, performed computational prediction of protein structure and molecular docking studies and wrote the manuscript

Sumudu R Perera performed laboratory experiments, analyzed data and wrote the manuscript

George S Katselis and **Paulos Chumala** performed Mass Spectrometry and Liquid Chromatography-Mass Spectrometry experiments and interpreted the results

Irene Martin supplied the *N. gonorrhoeae* 8903 strain containing the pJRD20 plasmid

Anthony Kusalik supervised Reema Singh and analyzed the data

Kristen M Mitzel performed laboratory experiments

Jo-Anne R Dillon conceptualized the research, provided guidance with the experimental design, feedback and editing on the manuscript and held the research grant that supported this work

6.1 ABSTRACT

Seven structurally related β-lactamase-producing plasmids have been characterized in penicillinase-producing *Neisseria gonorrhoeae* (PPNG) isolates. We characterized a variant (i.e. pJRD20, Canada-type) of the Africa-type (pJD5) plasmid isolated from *N. gonorrhoeae* strain 8903. The objective of this research was to compare the DNA sequence of pJRD20 with pJD5 and pJD4 (Asia type) and their TEM-1 β-lactamases. *N. gonorrhoeae* 8903 was identified as part of the Gonococcal Antimicrobial Surveillance Program in Canada. β-lactamase production was assessed using nitrocefin. MICs were determined by agar dilution and Etest methods (CLSI). The DNA sequences of pJRD20, pJD5 and pJD4 were assembled and annotated. The structure of TEM-1 and its penicillin-binding properties were determined by *in silico* molecular modelling and docking. TEM-1 proteins were characterized by Western blot, mass spectrometry and ampicillin hydrolysis assays. *N. gonorrhoeae* 8903 exhibited intermediate susceptibility to penicillin with

slow β-lactamase activity (i.e. 35 min to hydrolyze nitrocefin). Except for a novel 6 bp deletion starting at the G of the ATG start codon of *bla*$_{\text{TEM-1,}}$ the DNA sequence of pJRD20 was identical to pJD5. The TEM-1 β-lactamase produced by pJRD20 is 24 KDa and hydrolyzes ampicillin only after several hours. This unusual PPNG isolate might have been characterized as a non-PPNG due to its low MIC to penicillin and its very slow hydrolysis of nitrocefin. Given the unusual nature of its TEM-1 β-lactamase, laboratories might consider extending the length of time for nitrocefin hydrolysis assays.

6.2 INTRODUCTION

β-lactamase producing plasmids were first isolated from *N. gonorrhoeae* in 1975 and are one of the main causes of penicillin resistance in this organism (1). Penicillinase-producing plasmids of *N.gonorrhoeae* (PPNG) are named according to their first source of geographic isolation; i.e. Asia (7,426 bp), Africa (5,599 bp), Toronto/Rio (5,154 bp), Nimes (6,798 bp), New Zealand (9,309 bp), Johannesburg (4,865 bp) and Australia (3,269 bp) plasmid types (2, 3). Asia, Africa and Toronto/Rio plasmid types have been associated with epidemic outbreaks while the other plasmid types have been isolated sporadically (4). The structural diversity of β-lactamase-producing plasmids of *N. gonorrhoeae* is attributed to the presence of repeat sequences involved in DNA rearrangements, such as duplications and deletions (5). The plasmids are all related structurally to the Asia-type plasmid, with Africa-, Toronto/Rio-, and Johannesburg-types being unique deletion derivatives, the New Zealand-type plasmid an insertion derivative, and the Nimes-type plasmid both a deletion and insertion derivative (6-9).

In *N. gonorrhoeae*, most penicillinase-producing plasmids carry a $bla_{\text{TEM-1}}$, with $bla_{\text{TEM-135}}$ being the most common variant (1, 2, 7, 10-14). $bla_{\text{TEM-1}}$ is found in the ancestral Asia-type plasmid, while some strains of Asia-, Africa-, Toronto/Rio- and Australian-type plasmids can carry $bla_{\text{TEM-135}}$ (7, 15, 16). Diversity in TEM-1 β-lactamases arises through SNPs (14). TEM-135 differs from TEM-1 by one SNP (M182T, position 539 in the bla_{TEM} gene) (15, 16). TEM-220, isolated from Toronto/Rio-type plasmids, differs from TEM-135 by one additional SNP (A185T, position 547 in the bla_{TEM} gene) (3, 17). In addition, four novel amino acid substitutions (P14T, P14S, E110K, and G228S) have been identified in TEM of Asia- and Africa-type β-lactamase producing plasmids; however, allele numbers have not been assigned (7). With the selective pressure exerted by the use of extended-spectrum cephalosporins (ESC) to treat gonococcal infections, a concern is that $bla_{\text{TEM-135}}$ or $bla_{\text{TEM-220}}$ may acquire additional mutations thereby evolving to encode an ESBL (3, 17). Such an enzyme would be capable of hydrolyzing all ESC, including ceftriaxone, potentially leading to untreatable gonorrhea infections (7, 11, 14, 18, 19).

We have characterized a novel β-lactamase-producing plasmid from a strain of *N. gonorrhoeae* (i.e. 8903) with an intermediate MIC to penicillin and in which β-lactamase production is delayed

significantly. We compared the DNA sequence of this plasmid, which we named pJRD20 (Canada-type), to that of pJD4 (Asia-type) and pJD5 (Africa-type) and determined that pJRD20 is identical to pJD5 except for a 6 bp deletion starting at the G of the ATG start codon of $bla_{\text{TEM-1}}$ (2, 5). The DNA sequence of $bla_{\text{TEM-1}}$ and the structural features of the TEM-1 β-lactamase were investigated to ascertain the effects of the 6 bp deletion on protein expression and β-lactamase activity. We confirmed that a truncated 24 KDa TEM-1 penicillinase using an alternative downstream ATG start site was produced (i.e. TEM-$1_{\text{S-ATG}}$; where 'S' stand for short) and that this protein hydrolyzed ampicillin more slowly than the TEM-1 β-lactamase.

6.3 MATERIALS AND METHODS

6.3.1 Bacterial Strains and Plasmids

N. gonorrhoeae 8903 was isolated in 1992 as part of the Gonococcal Antimicrobial Surveillance Program (GASP) in Canada. WHO strains O and F were used as positive and negative controls for β-lactamase production respectively. *N. gonorrhoeae* F62 (20), WHO O and F were cultured on GC medium base (BD Bioscience, Mississauga ON, Canada) supplemented with 1% Kellogg's defined supplement (GCMBK) and incubated at 35-37°C with 5% CO_2 in a humid environment, for 18–24 h (21, 22). *N. gonorrhoeae* 8903 was grown on GCMBK supplemented with 0.125 mg/L ampicillin. *E. coli* strains DH5α (pJD4) and C600 (pJD5), previously transformed with pJD4 and pJD5, respectively, were grown overnight at 37^{o}C on LB agar with 10 mg/L ampicillin (23).

6.3.2 MIC Determination and Detection of β-Lactamase Activity

MICs and breakpoint interpretations of penicillin, tetracycline, spectinomycin, ceftriaxone, ciprofloxacin, erythromycin, gentamicin and azithromycin (Sigma-Aldrich, Milwaukee WI, USA) were ascertained using agar dilution and Etest (Biomerieux, St Laurent QC, Canada) as described by the CLSI (22). β-lactamase production was detected using nitrocefin discs (Sigma-Aldrich) and a liquid assay using nitrocefin powder (Sigma-Aldrich), as described by the manufacturer. The red colour indicated positive activity as compared to the control.

6.3.3 Plasmid Isolation and Sequencing

Plasmid DNA from *N. gonorrhoeae* 8903, grown overnight on GCMBK supplemented with 0.125 mg/L ampicillin, and *E. coli* strains DH5α (pJD4) and C600 (pJD5), which were grown overnight on LB agar in the presence of 10 mg/L ampicillin, was isolated using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, Edmonton AB, Canada). Plasmid content was verified by agarose gel electrophoresis and DNA concentration was measured using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). Plasmid DNA sequencing and assembly were performed at the CCIB (Center for Computational and Integrative Biology) DNA Core Facility at the Massachusetts General Hospital (MGH), Cambridge MA, USA. Briefly, Illumina compatible adapters with unique barcodes were ligated onto the sample during library construction. Libraries were sequenced on an Illumina MiSeq platform with 2x150 run parameters.

6.3.4 Transformation of *N. gonorrhoeae* and *E. coli* with pJRD20

For *N. gonorrhoeae* F62 transformations, pJRD20 DNA (1 µg) was mixed with recipient cells and liquid transformations were performed as described previously (24). *E. coli* DH5α transformations were modified as follows (25). Circular pJRD20 DNA was mixed with *E. coli* DH5α competent cells and heat-shocked at 42ºC for 30 seconds. Transformants were grown overnight at 37ºC on LB agar supplemented with 0.125 mg/L ampicillin. Plasmid DNA was extracted from *N. gonorrhoeae* and *E.coli* transformants as described above. The *bla*$_{TEM}$ genes were PCR amplified using *tem1* primers (synthesized at Invitrogen, Carlsbad CA, USA) (26) and amplicons were sequenced (Eurofins Genomics; Toronto ON, Canada) using the same primers.

6.3.5 DNA Sequence Analysis

Plasmids were assembled using MGH CCIB's *de novo* assembler UltraCycler v1.0. (Brian Seed and Huajun Wang, unpublished). Once the assembly outputs were manually inspected and passed quality control standards, results were downloaded. Full-length genomic sequences of pJD4, pJD5 and JRD20 assembled plasmids were aligned in Jalview (27) using the ClustalW algorithm (with default parameters) (28). The complete nucleotide sequence of *E.coli* KWB5 *bla*$_{TEM-1}$ (KY466951.1) was downloaded from the NCBI nucleotide database using the search term "*bla*$_{TEM-1}$". This sequence was aligned against pJD4 and pJD5 nucleotide sequences using the blastn program in NCBI-blast v2.6.0+ (default parameters) (29). The *bla*$_{TEM-1}$ hit regions from pJD4, pJD5 and pJRD20 were extracted using the extractseq command from EMBOSS-6.6.0 (30). Multiple sequence alignments for pJD4, pJD5 and pJRD20 *bla*$_{TEM-1}$ were performed in jalview using the ClustalW algorithm with default parameters. The aligned nucleotide sequences also included 100 bp upstream of *bla*$_{TEM-1}$. The *bla*$_{TEM-1}$ sequences on pJD5 and pJRD20 were examined both computationally and manually with DNAMAN software (v9 [with default parameters], Lynnon Corp). Computational prediction (31) identified an alternative GTG start codon (position 179; translated as fMet) (32) that would encode a truncated TEM. Since automatic genome annotations and gene prediction have limitations (33), the sequence was also examined manually and a second possible start codon, ATG at position 290 in pJRD20 *bla*$_{TEM-1}$ was identified and curated (34). The DNA sequences of pJD5 and pJRD20 with annotations have been submitted to Genbank under accession numbers MH140435 and MH140434 respectively.

ORFs were predicted using the getorf (with parameters "-table 11" and "-minsize 150") command from EMBOSS-6.6.0 (30). Functional annotations of predicted ORFs were performed using Blast2GO standalone version (35). To confirm the novelty of the 6 bp deletion in pJRD20, its TEM-1 β-lactamase sequence was aligned against all available (total 215) TEM β-lactamases (TEM numbers were matched with the list present at the Lahey website https://www.lahey.org/Studies/temtable.asp and at the NCBI ftp://ftp.ncbi.nlm.nih.gov/pathogen/betalactamases/Allele.tab). Allele sequences for all TEM β-lactamases were downloaded from the Pasteur database (http://bigsdb.pasteur.fr; last updated on 2013) and the NCBI protein database (https://www.ncbi.nlm.nih.gov/protein; accessed 2018_12).

6.3.6 Structural Analysis and Molecular Docking

Three-dimensional homology models of TEM-1 β-lactamase from pJD5 and pJRD20 were built using modeller v9.19 (36). For homology modelling, the best suitable template protein, 1esu (53) (*E. coli* TEM-1 β-lactamase mutant with a mutation at position 235 where Alanine replaces Serine [S235A]) was selected over other possible templates because of its better crystallographic resolution (2Å) and R factor (0.162). The TEM-1 sequences were used as queries against the Protein Databank (pdb) using blastp at the NCBI blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi?). The top hits showing more than 90% sequence identity with the TEM-1 β-lactamase were used to build a profile (build_profile.py) in modeller.

After examining the profile build outputs, the best template was selected based on the quality of crystallographic resolution. Following the construction of target-template alignments, homology models were built in modeller and the resulting structures were evaluated based on the best DOPE (Discrete Optimized Protein Energy) score. To assess the effect of novel deletion in pJRD20, the protein binding affinity of TEM-1 β-lactamase and molecular docking of the modelled structure with penicillin was performed *in silico* using AutoDockTools 4.2. (37) PubChem database (38) was used to download the Penicillin G (PNN) ligand ($C_{16}H_{18}N_2O_4S$). For molecular docking, both protein and ligand were prepared by adding hydrogens and computing Gasteiger charge. The docking parameters used for the docking modelling were Lamarckian genetic algorithm with population size (150), gene mutation rate (0.02), crossover rate (0.8) and the total number of runs (50). The grid box was centred over the ligand with the number of grid points in x=48, y=50 and

z=52 with a grid spacing of 0.375. Best docking pose from a set of predicted docking conformations was identified based on lower binding energy and inhibition constant.

6.3.7 Western Blot Analysis

N. gonorrhoeae 8903 (pJRD20), *N. gonorrhoeae* FA1090 (negative control), *E. coli* DH5α (pJD4), and *E. coli* C600 (pJD5) were collected from overnight growth on GCMBK or LB agar and lysed with RIPA lysis buffer (Santa Cruz Biotechnology, Mississauga, ON, Canada) in the presence of Halt protease and Halt Phosphatase inhibitors (Thermo Fisher). Protein concentrations were measured using a Pierce BCA Protein assay kit (Thermo Fisher) and volumes loaded onto 12% or 15% SDS-PAGE gels were modified accordingly. The membrane was incubated in an anti-β-lactamase monoclonal mouse primary antibody (specific for TEM β-lactamases; Abcam ab12251, Toronto ON, Canada) overnight at 4ºC, and subsequently in IRDye 800CW anti-mouse secondary antibody (Mandel Scientific LIC926-32210, Guelph ON, Canada) for 1 h. Bands were visualized using Odyssey CLX (LI-COR Mandel Scientific).

6.3.8 Protein Identification by Mass Spectrometry (MS)

Protein identification by mass spectrometry was done as described in Brandt *et al*. (39) Crude cell lysates from *N. gonorrhoeae* 8903 (pJRD20), *E. coli* DH5α (pJD4), *E. coli* C600 (pJD5) and *N. gonorrhoeae* FA1090 (negative control) were boiled with SDS. Proteins were separated on a 12% SDS-PAGE and the gel was stained with Coomassie dye. All chemicals for the MS analysis, unless noted otherwise, were purchased from Thermo Fisher Scientific. Protein bands corresponding to the area of interest were excised from the gel and destained twice with 100 µL of 200 mM ammonium bicarbonate (NH_4HCO_3) in 50% acetonitrile at 30°C for 20 minutes. Gel samples were then treated with acetonitrile for 10 minutes and dried with a speed-vac. Proteins were reduced with 100 µL of 10 mM dithiothreitol (DTT) in 100mM NH_4HCO_3 and incubated at 56°C for 1 hour. DTT was removed and replaced with 100 µL of 100 mM iodoacetamide and incubated at room temperature in the dark for 30 minutes. After washing twice with 200 mM NH_4HCO_3, samples were shrunk with acetonitrile, re-swelled with 200 mM NH_4HCO_3 and re-shrunk with acetonitrile. Samples were dried with a speed-vac and sequentially re-swelled in 20 µL trypsin buffer (50 ng/mL sequencing grade modified trypsin [Promega] in 1 mM hydrochloric acid and 100 mM NH_4HCO_3) and 30 µL of 200 mM NH_4HCO_3. Proteins were digested overnight at 30°C with shaking (300 rpm).

Trypsin action was quenched with 1% trifluoroacetic acid and tryptic peptides were extracted from gel slices in 100µL of 0.1% trifluoroacetic acid in 60% acetonitrile and stored at -80°C until MS analysis.

Extracted tryptic peptides were dried with a speed-vac and reconstituted in 12 µL of MS grade water:acetonitrile:formic acid (97:3:0.1 v/v). Insoluble material was removed by centrifugation at 18,000 x g for 10 minutes at 4ºC. A 10 µL aliquot was used for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Mass spectral analyses were performed on an Agilent 6550 iFunnel quadrupole time-of-flight (QTOF) mass spectrometer equipped with an Agilent 1260 series liquid chromatography instrument and an Agilent Chip Cube LC-MS interface (Agilent Technologies, Mississauga, ON, CA). Chromatographic peptide separation was accomplished using a high-capacity Agilent HPLC-Chip II: G4240-62030 Polaris-HR-Chip-3C18 consisting of a 360 nL enrichment column and a 75 µm × 150 mm analytical column, both packed with Polaris C18-A, 180Å, 3 µm stationary phase. Samples were loaded onto the enrichment column with 0.1% formic acid in water at a flow rate of 2.0 µL min^{-1}. After loading onto the analytical column, peptides were separated with a linear gradient solvent system consisting of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). The linear gradient was 3–25% solvent B for 50 minutes and then 25–90% solvent B for 10 minutes at a flow rate of 0.3 µL min^{-1}. Positive-ion electrospray mass spectral data were acquired using a capillary voltage set at 1900V, the ion fragmenter set at 360V, and the drying gas (nitrogen) set at 225°C with a flow rate of 12.0 L min^{1}. Spectral results were collected over a mass range of 250–1700 (mass/charge; m/z) at a scan rate of 8 spectra s^{-1}. MS/MS data were collected over a range of 50–1700 m/z and a set isolation width of 1.3 atomic mass units. A maximum of 20 precursor ions was selected for auto MS/MS at an absolute threshold of 3000 counts and a relative threshold of 0.01% with a 0.25-minute active exclusion.

MS/MS spectral data were extracted from raw data and processed against the concatenated SwissProt *Neisseria gonorrhoeae* database (UniProt release 2018_08), using Spectrum Mill (Agilent Technologies) as the database search engine. Search parameters included a fragment mass error of 50 ppm, a parent mass error of 20 ppm, trypsin cleavage specificity (two missed cleavages per peptide), and carbamidomethylation as a fixed modification of cysteine. Oxidized methionine,

carbamylated lysine, pyroglutamic acid, deamidated asparagine, phosphorylated serine, threonine, and tyrosine and acetyl-lysine were set as variable modifications. Data were also searched using semi-trypsin non-specific C- and N-terminus to enhance protein identification. Spectrum Mill validation was performed at peptide and protein levels (1% false discovery rate).

6.3.9 Liquid Chromatography (LC) – Mass Spectrometry (MS) Analysis

To examine ampicillin degradation by truncated TEM-$1_{S\text{-}ATG}$ in pJRD20 as compared to pJD5 TEM-1 β-lactamase, ampicillin hydrolysis assays were performed over a 6 h time course as described previously (54). Cleanup of the 20 µL cell supernatants was carried out by passing through a pre-conditioned C_{18} column (100 µL OMIX C_{18} pipette tip, Agilent Technologies Canada) followed by elution with 100 µL acetonitrile:water (50:50 v/v). The eluent was dried in a speed vac, reconstituted in 300 µL acetonitrile:water (3:97 v/v), and subjected to mass spectrometric analysis on an Agilent 6550 iFunnel quadrupole time-of-flight (QTOF) mass spectrometer equipped with an Agilent 1260 series liquid chromatography instrument and an Agilent Chip Cube LC-MS interface (Agilent Technologies Canada). Chromatographic separation was accomplished using a high-capacity Agilent HPLC-Chip II: G4240-62030 Polaris-HR-Chip-3C18 consisting of a 360 nL enrichment column and a 75 µm × 150 mm analytical column, both packed with Polaris C18-A, 180Å, 3 µm stationary phase. Samples were loaded onto the enrichment column with 0.1% formic acid in water at a flow rate of 2.0 µL min^{-1}. Separation occurred with a linear gradient solvent system consisting of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). The linear gradient was 3–11% solvent B for 15 min and then 11–20% solvent B for 4 min at a flow rate of 0.3 µL min^{-1}. Positive-ion electrospray mass spectral data were acquired using a capillary voltage set at 1750V, the ion fragmenter set at 360V, and the drying gas (nitrogen) set at 225ºC with a flow rate of 12.0 l min^{-1}. MS spectral data were collected over a mass range of 250–1700 (mass/charge; m/z) at a scan rate of 8 spectra s^{-1}. MS/MS data were collected over a range of 50–1700 m/z and a set isolation width of 1.3 atomic mass units. The collected MS spectral data were analyzed using the MassHunter qualitative analysis software (version B.07.00; Agilent Technologies Canada).

6.4 RESULTS

6.4.1 An Intermediate MIC and Slow β-Lactamase Activity Observed in pJRD20.

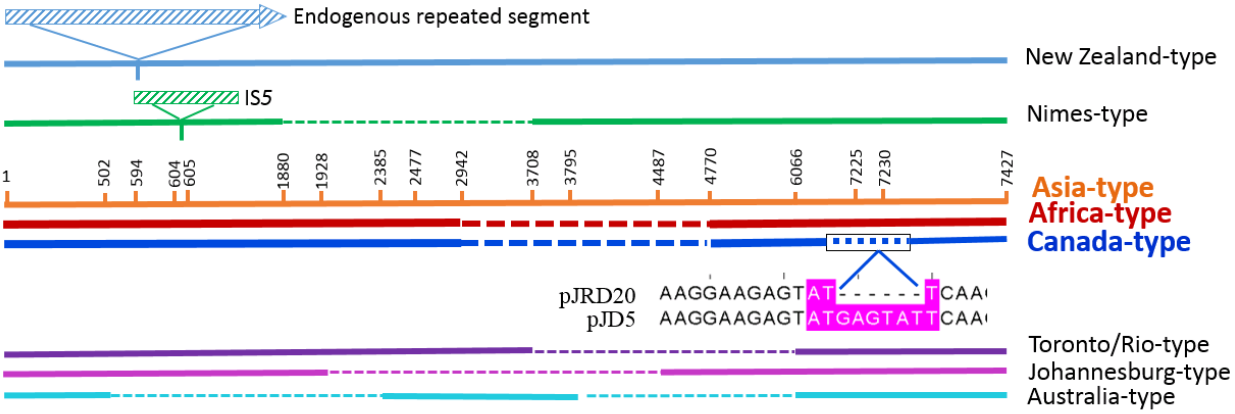
The median MICs against *N. gonorrhoeae* 8903 harboring pJRD20 were (mg/L): penicillin 0.125, tetracycline 0.5, ciprofloxacin 0.003, ceftriaxone <0.016, azithromycin 0.047, erythromycin 0.19, spectinomycin 12, and gentamicin 4.

The positive control *N. gonorrhoeae* WHO O produced a proper colour reaction (yellow to red) instantaneously when tested for β-lactamase activity using nitrocefin. *N.gonorrhoeae* WHO F (negative control) did not show β-lactamase activity. *N. gonorrhoeae* 8903 was slowly positive for β-lactamase activity, 35 minutes. When *E. coli* DH5α was transformed with pJRD20, slow β-lactamase activity (≥4 h) was observed as compared to *N. gonorrhoeae* 8903. *N. gonorrhoeae* F62 transformed with pJRD20 resulted in significantly slower β-lactamase activity (>6 h).

6.4.2 pJRD20 Has a Novel 6 bp Deletion Starting at the G of the ATG Start Codon of *bla*$_{\text{TEM-1}}$

As compared to pJD4, an identical deletion of 1828bp (co-ordinates 2942–4770 bp in pJD4; Figure 6.1A) is present in both pJRD20 and pJD5 (5). In addition, pJRD20 has a novel 6 bp deletion from position 5389 to 5394 bp (co-ordinates 7225–7230 bp [GAGTAT] in pJD4; encodes Met, Ser, and Ile; Figure 6.1). The *bla*$_{\text{TEM-1}}$ regions were extracted from assembled plasmid sequences and the location of the 6 bp deletion was confirmed through multiple sequence alignments of *bla*$_{\text{TEM-1}}$ from the three plasmids. Multiple sequence alignment of the pJRD20 *bla*$_{\text{TEM-1}}$ sequence with *bla*$_{\text{TEM-1}}$ (KY466951.1), *bla*$_{\text{TEM-135}}$ (GQ896333.1) and *bla*$_{\text{TEM-220}}$ (KM998962) sequences confirmed that pJRD20 carries *bla*$_{\text{TEM-1}}$ with a 6 bp deletion which starts at the G of the ATG start codon. To confirm the novelty of the pJRD20 6 bp deletion at the protein level, the pJRD20 TEM-1 β-lactamase was compared with the publicly available list of 215 TEM β-lactamase sequences (Lahey clinic website and NCBI). The novel 6 bp deletion in pJRD20 TEM-1 β-lactamase has not been reported previously in any other TEM β-lactamase. Multiple sequence alignment of the pJRD20 TEM-1 β-lactamase with TEM-1 (NP_052173.1), TEM-135 (ACX46122.1) and TEM-220 (AIW68620.1)[3] β-lactamases confirmed that pJRD20 encodes TEM-1.

A) Linear alignment map of the gonococcal β-lactamase producing plasmids



B) Multiple sequence alignment of pJRD20 TEM-$1_{S\text{-}ATG}$ and pJD5 TEM-1

```
pJRD20_TEM-1S-ATG    1 ----------------------------------------------------------MMSTFKVLL  9
pJD5_TEM-1           1 MSIQHFRVALIPFFAAFCLPVFAHPETLVKVKDAEDQLGARVGYIELDLNSGKILESFRPEERFPMMSTFKVLL 74

pJRD20_TEM-1S-ATG   10 CGAVLSRVDAGQEQLGRRIHYSQNDLVEYSPVTEKHLTDGMTVRELCSAAITMSDNTAANLLLTTIGGPKELTA 83
pJD5_TEM-1          75 CGAVLSRVDAGQEQLGRRIHYSQNDLVEYSPVTEKHLTDGMTVRELCSAAITMSDNTAANLLLTTIGGPKELTA 148

pJRD20_TEM-1S-ATG   84 FLHNMGDHVTRLDRWEPELNEAIPNDERDTTMPAAMATTLRKLLTGELLTLASRQQLIDWMEADKVAGPLLRSA 157
pJD5_TEM-1         149 FLHNMGDHVTRLDRWEPELNEAIPNDERDTTMPAAMATTLRKLLTGELLTLASRQQLIDWMEADKVAGPLLRSA 222

pJRD20_TEM-1S-ATG  158 LPAGWFIADKSGAGERGSRGIIAALGPDGKPSRIVVIYTTGSQATMDERNRQIAEIGASLIKHW          221
pJD5_TEM-1         223 LPAGWFIADKSGAGERGSRGIIAALGPDGKPSRIVVIYTTGSQATMDERNRQIAEIGASLIKHW          286
```

Figure 6.1 pJRD20 Canada-type in comparison to other gonococcal β-lactamase producing plasmids A) Linear alignment map of the gonococcal β-lactamase producing plasmids. Coordinates are based on the Asia-type plasmid pJD4. Deletions are highlighted with dashed lines. The novel 6bp deletion within the ATG start-site of pJRD20 *bla*$_{TEM\text{-}1}$ is highlighted with dashes in the pink colour box (pJD5 and pJRD20 alignment). B) Protein multiple sequence alignment of pJRD20 TEM-$1_{S\text{-}ATG}$ and pJD5 TEM-1 were generated using the Muscle algorithm and visualized in Jalview. Truncated N-terminal residues are highlighted in the blue box.

A total of 29 direct repeats are present in pJRD20 (13 on the forward strand and 16 on the reverse strand; Table 6.1). The longest direct repeat is 43 bp, followed by two 27 bp repeats, one 22 bp repeat, and two 21 bp repeats. The remaining repeats are <20 bp long. Furthermore, 27 inverted repeats are present in pJRD20. pJRD20 carries 38 ORFs (both strands) with a minimum of 50 AA (150 nucleotides; Table 6.1). Functions were assigned to the putative ORFs by searching the NCBI protein database using Blast2GO. ORFs were annotated as DNA strand transferase (2194-686 bp), recombinase (487-2 bp), TEM-1 (5398-4535 bp), replication protein (3169-4227 bp), transposase for transposon Tn3 (2-166 bp) (1, 5), integrase (170-409 bp) and hypothetical proteins (75-278, 4929-5102, 5546-5292bp).

6.4.3 The 6 bp Deletion Leads to a Truncated 24 KDa TEM-1$_{S\text{-}ATG}$ β-Lactamase With Altered Penicillin-Binding Properties

In silico examination of the pJRD20 *bla*$_{TEM\text{-}1}$ coding sequence indicated that *bla*$_{TEM\text{-}1}$ could be translated into two different truncated proteins (i.e. TEM-1$_{S\text{-}ATG}$ and TEM-1$_{S\text{-}GTG}$; Figure 6.2,). In option 1, the first 65 N-terminal residues would be eliminated from TEM-1 (i.e. TEM-1$_{S\text{-}ATG}$) and the start codon for translation in *bla*$_{TEM\text{-}1}$ would be an ATG 663 bp downstream (Figure 6.2A). In option 2, 28 residues would be deleted from the N-terminus, with a start codon of GTG (Figure 6.2B; i.e. TEM-1$_{S\text{-}GTG}$).

Molecular docking studies of the proposed truncated proteins with penicillin indicated altered protein-ligand interactions for both truncated proteins as compared to TEM-1. Penicillin binds at pJD5 TEM-1 active site by interacting with a serine residue at position 68 by forming hydrogen bonds with a distance (and energy) of 1.787 (-0.039), 1.762 (-2.382) and 2.116 (-4.416) with arginine at position 241 (Figure 6.3A). Whereas in pJRD20 TEM-1$_{S\text{-}ATG}$, penicillin binds with allosteric sites other than the serine active site residues (Figure 6.3B). Arginine at position 124 interacts with penicillin by making a hydrogen bond with distance 2.106 (and energy -0.991). The binding energy and inhibition constant of this truncated protein are smaller (although larger than pJD5 TEM-1) as compared to the other hypothetical TEM-1$_{S\text{-}GTG}$ in figure 6.3C. In the case of TEM-1$_{S\text{-}GTG}$ (figure 6.3C), instead of serine, penicillin interacts with lysine at position 185 by making a hydrogen bond (distance = 2.064 and energy = -0.161). In the case of pJD5, the penicillin ligand binds to the TEM-1 serine active site with binding energy (a lower value indicates stable binding) of –6.14 and an inhibition constant (the stronger the protein-ligand interaction, the lower

Table 6.1 Comparison of the molecular characteristics of different β-lactamase producing plasmids of *N. gonorrhoeae*.

[a]Plasmid Type	[b]Genbank Accession Number	General Information		[c]Repeats		ORFs	*bla*TEM variants	Ref
		Length (bp)	%GC	Direct	Inverted	>=50		
Asia	U20374	7427	38.35	37	50	50	*bla*TEM-1	5
Africa	MH140435	5597	39.66	29 (<50bp)	27	38	*bla*TEM-1	2
Canada	MH140434	5591	39.67	29 (<50bp)	27	38	*bla*TEM-1S-ATG	This research
Toronto	NC_010881	5161	38.35	39	34	35	*bla*TEM-1	50
Johannesburg	NC_019211	4865	40.55	22	32	31	*bla*TEM-1	51
Australia	NC_025191	3269	41.69	6 (<=50bp)	8	23	*bla*TEM-135	15

[a]The complete DNA sequences for Nimes[8] and New Zealand[9] plasmid types are not available at NCBI.
[b]The DNA sequence of pJRD20 was compared with the nucleotide sequences of other *N. gonorrhoeae* β-lactamase producing plasmids.
[c]Direct and inverted repeats in pJD5 and pJRD20 nucleotide sequences were analyzed using RepEx software.[52]

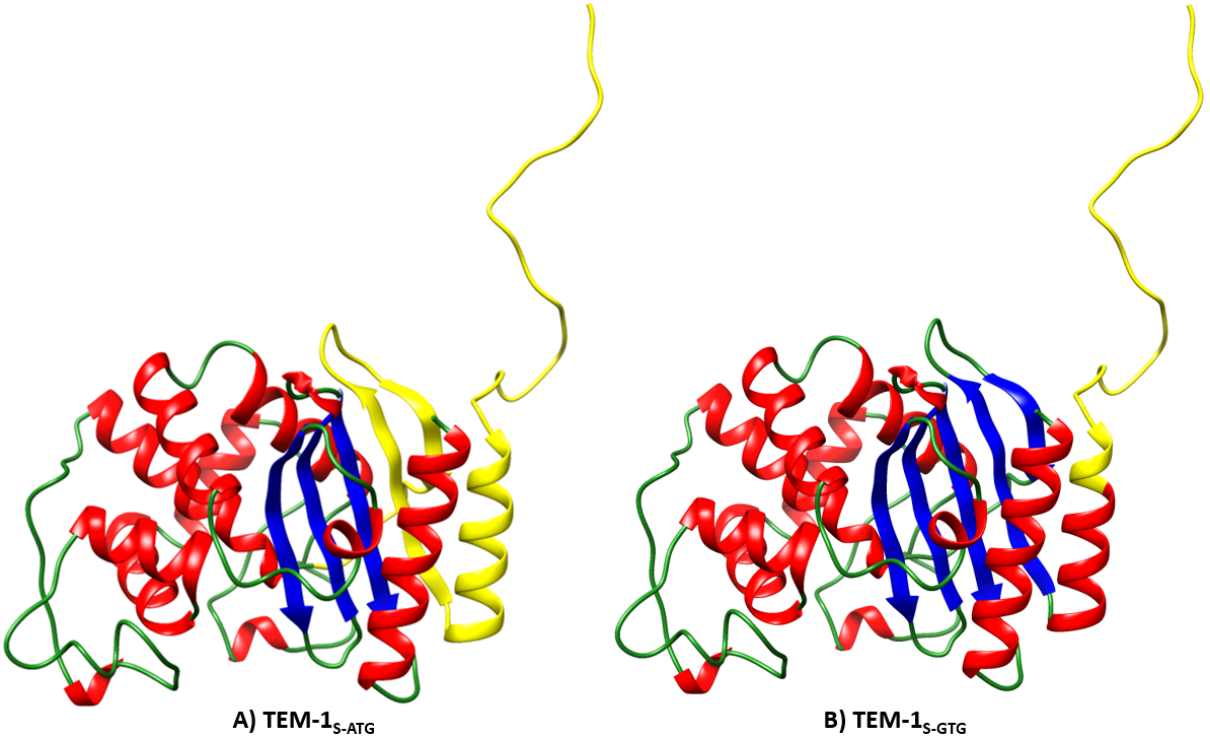


Figure 6.2 Homology modelling for the two predicted truncated TEM-1 proteins named TEM-$1_{S\text{-}ATG}$ and TEM-$1_{S\text{-}GTG}$. A) TEM-1 structure (Helix (red-color), β-sheet (blue color) and coil (dark green color)). The missing N-terminal 65 residues (in predicted TEM-$1_{S\text{-}ATG}$) involved in the formation of one helix, and two β-sheets and one loop leading into the core helix, are highlighted in yellow. B) The missing 28 residues (TEM-$1_{S\text{-}GTG}$) are shown in yellow.

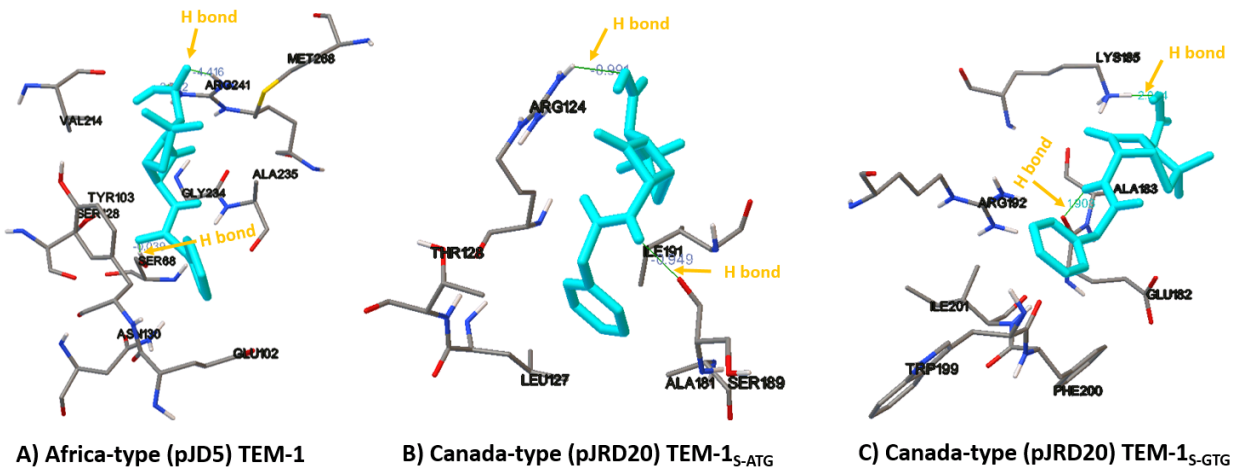


Figure 6.3 Protein-ligand interaction residues present at binding sites in pJD5 and pJRD20. The green dotted line represents the visible hydrogen bond (highlighted with orange arrows). Penicillin G ligand molecule is highlighted in cyan. A) Africa-type (pJD5) TEM-1; B) Canada-type (pJRD20) TEM-1$_{S\text{-}ATG}$; and C) Canada-type (pJRD20) TEM-1$_{S\text{-}GTG}$.

the inhibition constant) of 31.75 uM (Figure 6.3A). In the case of proposed truncated TEM-$1_{S\text{-}ATG}$ of pJRD20, the penicillin ligand would bind at the allosteric site with a binding energy and inhibition constant of –5.74 and 61.98 uM, respectively (Figure 6.3B). The proposed truncated TEM-$1_{S\text{-}GTG}$ would have a binding energy and inhibition constant of –5.3 and 130.52 uM, respectively (Figure 6.3C). These results indicated that the TEM-$1_{S\text{-}ATG}$ would produce a protein with more stable binding with its ligand. Therefore, we hypothesized that TEM-$1_{S\text{-}ATG}$ was produced from pJRD20.

6.4.4 Confirmation of Production of a 24 KDa TEM-$1_{S\text{-}ATG}$ β-Lactamase by Western Blot and MS Analyses

Western blot analysis showed that a truncated protein of 24 KDa (Figure 6.4, i.e. TEM-$1_{S\text{-}ATG}$) was produced by pJRD20, as predicted if the downstream ATG start site was used (Figure 6.4). The TEM-1 β-lactamase produced by pJD4 and pJD5 were 32 KDa, corresponding to the wild-type gonococcal TEM-1 β-lactamase size. These results were confirmed by Mass Spectrometry (MS). TEM-$1_{S\text{-}ATG}$ (24 KDa) was produced by pJRD20, whereas TEM-1 (32 KDa; wild-type) was produced by pJD5 (Table 6.2). The main difference in the peptide sequences of TEM-1 (32 KDa) and TEM-$1_{S\text{-}ATG}$ (24 KDa) is in the N-terminus sequence region between amino acids 1 and 65, which are missing from the latter (Figure 6.5). Four tryptic peptides identified from that region were present in the protein band excised from TEM-1 (32 KDa) and absent in the protein band excised from TEM-$1_{S\text{-}ATG}$ (24 KDa; Table 6.3). All other tryptic peptides identified in the TEM-$1_{S\text{-}ATG}$ (24 KDa) were identical to those identified in TEM-1 (32 KDa) between amino acids 70 and 290.

6.4.5 Ampicillin Hydrolysis by pJRD20 TEM-$1_{S\text{-}ATG}$

The LC-MS analysis of the hydrolysis assay after 6 h incubation showed that ampicillin degradation by pJRD20 TEM-$1_{S\text{-}ATG}$ was slow as compared to pJD5 TEM-1 (Table 6.4; Figure 6.6). The peak at approximately 13.5 minutes, in Figure 6.6A-E, corresponds to intact (non-hydrolyzed) ampicillin ($[M+H]^+$=350.1173). The two peaks at approximately 8.5 and 9.5 minutes in Figure 6.6C and 6.6D, respectively have the same mass (m/z 368.1318), which corresponds to the hydrolyzed form of ampicillin and also gave identical MS/MS fragmentations (m/z 175.0872, m/z 279.1194, m/z 307.1102 and m/z 324.1377; the latter corresponds to the hydrolyzed

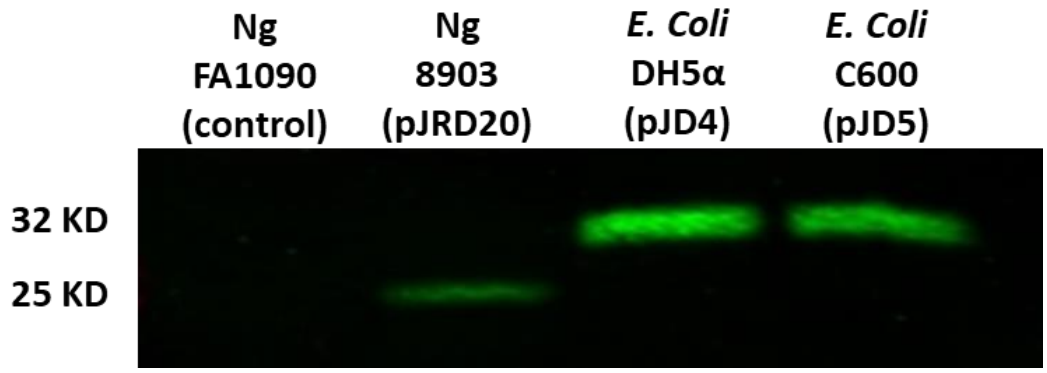


Figure 6.4 Western blot showing pJD4, pJD5 and pJRD20 TEM-1 proteins. The membrane was incubated in an anti-β-lactamase monoclonal mouse primary antibody (speficic for TEM β-lactamases).

Table 6.2 Proposed TEM-1 β-lactamase.

TEM-1 type	Name	Size (KD)	Length (AA)	Binding Energy
TEM-1 pJD5	Wild type TEM-1	32	286	-6.14
TEM-1 pJRD20 (ATG start)	$TEM\text{-}1_{S\text{-}ATG}$	24	221	-5.74

A) pJD5 *bla*$_{TEM-1}$ (290 aa, 32.02 KD)

```
1       N-term   K R K S M  S I Q H F  R V A L I  P F F A A  F C L P V  F A H P E  T L V K V  K D A E D     40
41     Q L G A R  V G Y I E  L D L N S  G K I L E  S F R P E  E R F P M  M S T F K  V L L C G  A V L S R     85
86     V D A G Q  E Q L G R  R I H Y S  Q N D L V  E Y S P V  T E K H L  T D G M T  V R E L C  S A A I T    130
131    M S D N T  A A N L L  L T T I G  G P K E L  T A F L H  N M G D H  V T R L D  R W E P E  L N E A I    175
176    P N D E R  D T T M P  A A M A T  T L R K L  L T G E L  L T L A S  R Q Q L I  D W M E A  D K V A G    220
221    P L L R S  A L P A G  W F I A D  K S G A G  E R G S R  G I I A A  L G P D G  K P S R I  V V I Y T    265
266    T G S Q A  T M D E R  N R Q I A  E I G A S  L I K H W   C-term                                      290
```

B) pJRD20 TEM-1$_{S-ATG}$ (Start codon – ATG – 221 aa, 24.13 KD)

```
1       N-term   M M S T F  K V L L C  G A V L S  R V D A G  Q E Q L G  R R I H Y  S Q N D L  V E Y S P     40
41     V T E K H  L T D G M  T V R E L  C S A A I  T M S D N  T A A N L  L L T T I  G G P K E  L T A F L     85
86     H N M G D  H V T R L  D R W E P  E L N E A  I P N D E  R D T T M  P A A M A  T T L R K  L L T G E    130
131    L L T L A  S R Q Q L  I D W M E  A D K V A  G P L L R  S A L P A  G W F I A  D K S G A  G E R G S    175
176    R G I I A  A L G P D  G K P S R  I V V I Y  T T G S Q  A T M D E  R N R Q I  A E I G A  S L I K H    220
221    W          C-term                                                                                221
```

Figure 6.5 pJD5 TEM-1 (a) and computationally predicted pJRD20 TEM-1$_{S-ATG}$ (b) protein sequences.

Table 6.3 Tryptic peptides identified in TEM-1 (32 KD) but not in TEM-$1_{S\text{-}ATG}$ (24 KDa).

Sequence	**Observed Mass**	**Actual MH^+**	**Charge**	**Peptide Score**
(K)VKDAEDQLGAR(V)	401.210	1201.617	3	11.6
(K)DAEDQLGAR(V)	487.732	974.454	2	13.8
(R)VGYIELDLNSGK(I)	654.347	1307.684	2	14.1
(K)ILESFRPEER(F)	425.897	1275.669	3	9.33

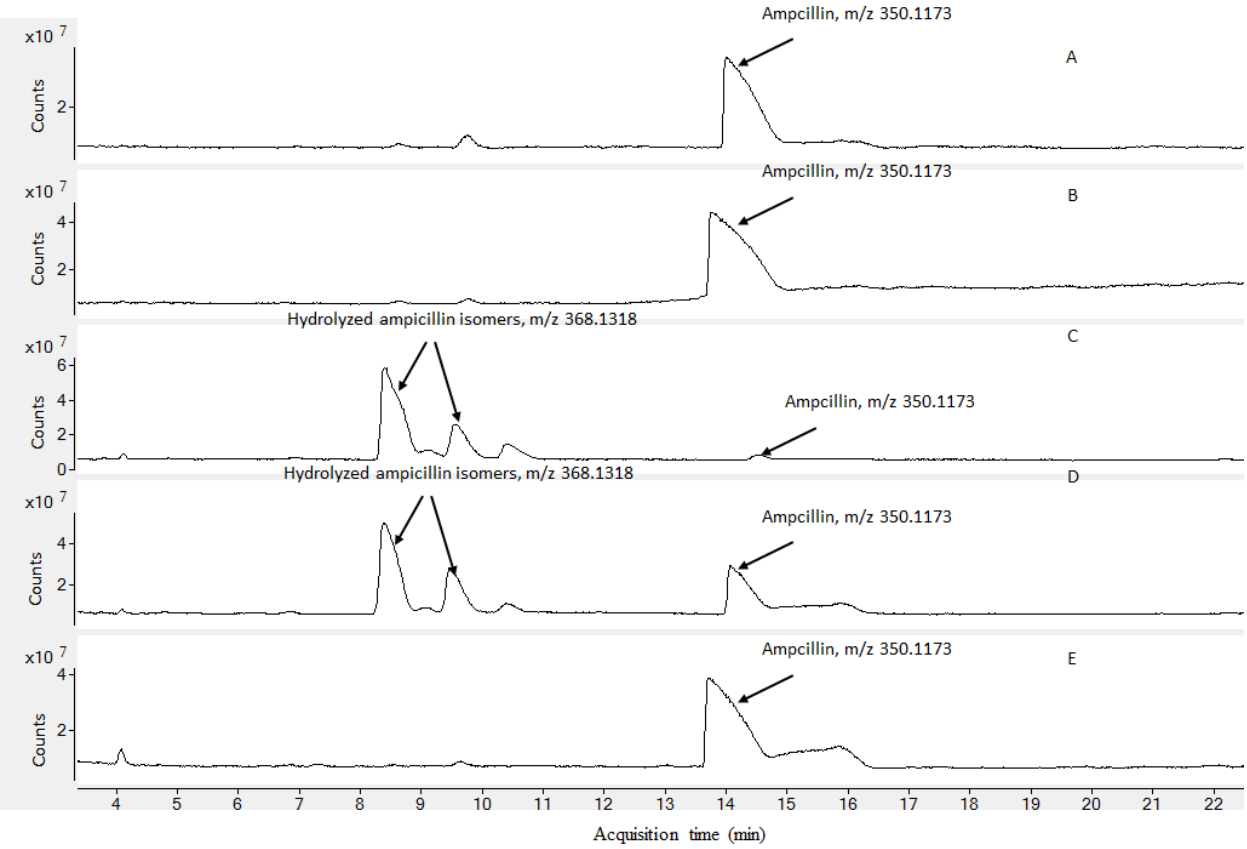


Figure 6.6 Total ion chromatograms (TIC), after 6-hour incubation, for ampicillin (A), ampicillin + *N. gonorrhoeae* WHO-F (B), ampicillin + *E. coli* c600 containing pJD5 (C), ampicillin + *N. gonorrhoeae* 8903 containing pJRD20 (D) and ampicillin + *N. gonorrhoeae* F62 containing pJRD20 (E)

decarboxylated form of ampicillin). These two peaks were identified as isomers of hydrolyzed ampicillin. Products of hydrolyzed ampicillin were only observed in *E. coli* c600 containing pJD5 and *N. gonorrhoeae* 8903 containing pJRD20. While pJD5 TEM-1 hydrolysis of ampicillin was instantaneous, even partial hydrolysis (~50%) by pJRD20 TEM-$1_{S\text{-}ATG}$ took up to 6 h (Table 6.4). *N. gonorrhoeae* F62 transformed with pJRD20 took upwards of 6 h for β-lactamase production, as measured by the ampicillin hydrolysis assay (Figure 6.6; Table 6.4) and nitrocefin degradation.

Table 6.4 LC-MS analysis of cell supernatant from the ampicillin hydrolysis assay.

Experiment	**Hydrolysis assay of ampicillin**	**Ampicillin Status (after 6 h incubation)**	**Mass of intact ampicillin and/or its hydrolysis products detected in the supernatant**
A	Ampicillin (10µg/µL, Positive control)	Not hydrolyzed (≈ 0%)	$[M+H]^+$= 350.1173
B	Ampicillin + *N. gonorrhoeae* WHO-F (Negative control)	Not hydrolyzed (≈ 0%)	$[M+H]^+$= 350.1173
C	Ampicillin + *E. coli* c600 (pJD5)	Completely hydrolyzed (100%)	$[M_{hyd}+H]^+$= 368.1318, $[M_{hyd/decarb}+H]^+$= 324.1388
D	Ampicillin + *N. gonorrhoeae* 8903 (pJRD20)	Partially hydrolyzed (≈ 50%)	$[M+H]^+$= 350.1173, $[M_{hyd}+H]^+$= 368.1318, $[M_{hyd/decarb}+H]^+$= 324.1388
E	Ampicillin + *N. gonorrhoeae* F62 transformed with pJRD20	Not hydrolyzed (≈ 0%)	$[M+H]^+$= 350.1173
F	H_2O (control) + *N. gonorrhoeae* 8903 (pJRD20)	-	-

6.5 DISCUSSION

A novel β-lactamase producing plasmid, pJRD20, from *N. gonorrhoeae* strain 8903 was sequenced and assembled. This plasmid was found to be identical to the Africa-type plasmid, pJD5, from *N. gonorrhoeae,* except for a 6 bp deletion starting at the G of the ATG start codon of $bla_{\text{TEM-1.}}$ An intermediate MIC to penicillin and slow hydrolysis of ampicillin were characteristic of *N. gonorrhoeae* 8903.

Functional analysis of the putative ORFs of pJRD20 indicated their involvement in biological functions such as DNA-directed DNA polymerase, lyase, β-lactamase and recombinase activities. Similar to pJD5, the Africa-type plasmid, pJRD20 has one replication protein, indicative of one origin of replication (*ori1*) (2). Previous research (2) indicated that pJD4, the Asia-type plasmid, contains three distinct origins of replication (*ori1*, *ori2*, and *ori3*), whereas the pJD5 has a single origin of replication (*ori1*).

We hypothesized that the 6 bp deletion starting at the G of the ATG start codon of $bla_{\text{TEM-1}}$ in pJRD20 altered the final protein structure. This novel deletion led to the formation of a truncated TEM-1 (TEM-$1_{\text{S-ATG}}$) with an alternate ATG start site. In screening TEM-$1_{\text{S-ATG}}$ from pJRD20 against all listed TEM β-lactamases, we determined that the 6 bp deletion has not been reported previously. Based on our molecular modelling and docking studies, we proposed that the 6bp deletion in pJRD20 $bla_{\text{TEM-1}}$ would lead to the loss of critical residues from the N-terminus but not the active site of the truncated TEM-$1_{\text{S-ATG}}$ (Figure 2). This would alter the penicillin-binding pattern of the protein. This hypothesis was supported by Western blot and mass spectrometry analyses, which indicated that a 24 KDa truncated protein (i.e. TEM-$1_{\text{S-ATG}}$) is produced by pJRD20. Furthermore, the β-lactamase hydrolysis assay confirmed the slow hydrolysis pattern of ampicillin.

This is the first report confirming that a 6 bp deletion starting at the G of the ATG start codon of $bla_{\text{TEM-1}}$ shifted the start codon to an alternate downstream ATG in *N. gonorrhoeae*. The examined truncated protein has a traditional ATG start codon and better binding affinity than the predicted truncated TEM-$1_{\text{S-GTG}}$ with a putative GTG start codon. This second protein was not produced.

Other proteins have been expressed from alternative start codons in truncated forms. One example is the translation of *xopAE* (encodes E3 ubiquitin ligase; translated using ATG) into a truncated protein using an alternate downstream ATT start codon in *Xanthomonas euvesicatoria* (40). Plasmids carrying *infB* (IF2α and IF2ß) (41), *clpB* (ClpB) (42), and *folC* (Folylpolyglutamate Synthetase–Dihydrofolate Synthetase) (43) genes with alternative downstream GTG start sites were constructed to measure the expression of the respective truncated proteins. These genes were translated into two proteins, a fully mature product encoded from "ATG" start codon and a truncated form-encoded from a downstream in-frame "GTG" start site. Truncated proteins in these studies showed varying functionality as compared to the mature protein. Rodas *et al* (44) reported that the translation of *N. gonorrhoeae narE* from a downstream GTG codon resulted in a truncated protein carrying a 49 amino acid deletion at the N-terminal. NarE shared 100% similarity with the NarE of *N. meningitis* (protein translated using ATG start codon). Furthermore, alternative downstream ATG start sites can be used to form truncated proteins (45, 46). Loh *et al*. described the formation of one mature $NolA_1$ and two N-terminally truncated functional proteins ($NolA_2$ and $NolA_3$) from *nolA*, thereby describing translation using three different ATG start codons in *Bradyrhizobium japonicum* (45). The $TipA_S$ (144 aa) is translated from an alternative ATG start site within *tipA* in *Streptomyces lividans* (46). Ours is the first report of a truncation in the TEM-1 β-lactamase with an alternative ATG start site downstream of the original start site in *N. gonorrhoeae*.

The β-lactamase is exported through the cytoplasmic membrane into the periplasm using its N-terminus signal peptide (47). In *E. coli* deletion mutants lacking the TEM signal sequence, the "RTEM" β-lactamase was maintained in the cytoplasm but not secreted (48). The excision of the signal sequence of *bla* did not affect the catalytic activity of "RTEM" β-lactamase (48). A 28 KDa truncated $bla_{TEM\text{-}1}$ lacking its signal sequence was constructed to study protein export in a mutant strain of *M. tuberculosis* and β-lactamase activity was detected using nitrocefin (49). β-lactam resistance was conferred when this truncated $bla_{TEM\text{-}1}$ was fused with either Sec or Tat export signal sequences (49). In the present research, the slow β-lactamase activity and protein expression detected using Western blotting indicated that pJRD20 is producing a 24 KDa truncated $TEM\text{-}1_{S\text{-}ATG}$. We suggest that the truncated $TEM\text{-}1_{S\text{-}ATG}$ (lacking the signal peptide) remains mostly in the cytoplasm, resulting in the loss of export to the periplasm and delayed hydrolysis of

penicillin/ampicillin. Interestingly, this isolate did not confer elevated resistance to penicillin, typical of β-lactamase-producing gonococci. However, further research will confirm whether the truncated protein is present in the cytoplasm or uses a novel mechanism to be exported across the membrane. Alternatively, the TEM-$1_{S\text{-}ATG}$ β-lactamase may be released from gonococci upon cell lysis.

In conclusion, we identified and characterized an Africa-type pJD5 variant plasmid, Canada-type pJRD20, with a 6 bp novel deletion starting at the G of the ATG start codon of $bla_{TEM\text{-}1}$. The plasmid showed an intermediate MIC to penicillin and slow β-lactamase production. *In silico* analysis showed the effects of the 6 bp deletion on the 3D TEM-1 structure and penicillin-binding patterns. Western blot and mass spectrometry analyses confirmed the production of a truncated TEM-$1_{S\text{-}ATG}$ sequence. This research highlights the importance of extended monitoring for β-lactamase activity when using nitrocefin, as these slow β-lactamase producing PPNG strains could easily be overlooked. Findings of novel plasmids harbouring bla_{TEM} variants, such as pJRD20, is of critical importance for the proper control and management of global antibiotic resistance in *N. gonorrhoeae*.

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6.8 TRANSPARENCY DECLARATIONS

None to declare.

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CHAPTER 7: CONCLUSIONS AND FUTURE CONSIDERATIONS

7.0 THE NEED FOR A RAPID DIAGNOSTIC TEST

N. gonorrhoeae surveillance calls for increased attention for early detection and screening strategies (especially in asymptomatic cases), partner management and development of new diagnostics to achieve a 90% reduction in gonorrhea incidence by 2030 as compared to the number of cases in 2018 (2). Molecular surveillance can enhance gonococcal diagnosis, monitor AMR, improve treatment capabilities and strengthen the knowledge gap in antimicrobial use and AMR mechanisms, leading to effective use of antimicrobials, increasing their longevity and the global control of AMR in gonococci (1, 3).

For my research, I developed a multiplex real-time PCR assay for the simultaneous identification of *N. gonorrhoeae* and its ciprofloxacin susceptibility status. Such a test could re-introduce ciprofloxacin for the treatment of susceptible gonococcal isolates. In addition, I evaluated a commercial hydrogel platform for the diagnosis of *N. gonorrhoeae* by using *N. gonorrhoeae* specific diagnostic primers and multiplex primers discriminating ciprofloxacin susceptible and resistant isolates. Preliminary results from these tests will lay the basis for commercial development of a portable diagnostic test for *N. gonorrhoeae*, possibly for use in resource-limited settings. Furthermore, I used molecular techniques and predicted trends of AMR in *N. gonorrhoeae* isolates from Saskatchewan. This showed emerging resistance to cefixime and azithromycin in Saskatchewan. Finally, I characterized a novel resistance-conferring mutation in a β-lactamase (*bla*) producing plasmid of an *N. gonorrhoeae* isolate, leading to slow β-lactamase production. This finding highlights the importance of continuous surveillance for *bla* plasmids and novel AMR mechanisms in *N. gonorrhoeae*.

7.1 SIMULTANEOUS IDENTIFICATION OF *N. GONORRHOEAE* AND ITS CIPROFLOXACIN SUSCEPTIBILITY PROFILE

Historically, a majority of *N. gonorrhoeae* isolates in Saskatchewan have been susceptible to ciprofloxacin and resistance has remained below 10% for many years (4). Due to the high prevalence of ciprofloxacin susceptible isolates in Saskatchewan, this antibiotic could be re-

introduced for treatment of susceptible isolates, provided that the susceptibility status of the isolate is known. In fact, the current treatment guidelines in the United Kingdom recommend ciprofloxacin therapy for susceptible *N. gonorrhoeae* isolates (5). Both commercial (6) and experimental (7-11) molecular tests have been implemented in the United States and elsewhere, in clinical settings, with high success rates. However, such a test is still needed in Canada and for resource-constrained settings.

For my research objective to develop rapid techniques for *N. gonorrhoeae* diagnosis, three primers were designed to work in multiplex to identify *N. gonorrhoeae* along with SNPs in its *gyrA* locus encoding S91 and D95 amino acid substitutions. These primers are unique in their capability to simultaneously identify *N. gonorrhoeae* and predict an isolate's ciprofloxacin susceptibility profile. For the first part of the research (Chapter 3), *gryA* multiplex primers were tested using *N. gonorrhoeae* cultures and were over 99% sensitive and specific. Subsequently, the performance of the primers using DNA from clinical specimens (urines, raw urines and remnant Aptima urine specimens) was assessed (Chapter 4). Although the sensitivity and specificity were not as high as with cultures, the *gyrA* multiplex primers had 80% sensitivity with DNA extracted from urine specimens. As the ultimate goal of this research is to develop rapid diagnostic tools for *N. gonorrhoeae* identification, these multiplex primers were incorporated into a portable hydrogel-based RT-PCR platform (Chapter 4). Despite the limited number of specimens, this preliminary test shows future potential in the amalgamation of the *gryA* multiplex primers on to the hydrogel system to develop a portable diagnostic platform for *N. gonorrhoeae*.

The low sensitivities observed with raw urine specimens could be due to a variety of contributing factors. Modification of the PCR parameters (i.e. SYBR concentration, number of cycles, the volume of sample, etc.) to better suit the given specimen (i.e. raw urine) may prove more useful. RT-PCR conditions were initially validated with *N. gonorrhoeae* cultures. However, in hindsight, we used the same conditions to test clinical specimens and did not perform extensive validation on urines or remnant Aptima urine specimens prior to experiments with these specimens. Therefore, re-validating the PCR conditions for urines and changing one parameter at a time to better adapt the RT-PCR protocol to our sample would provide promising results. Furthermore, diluting the urine specimens may abrogate PCR inhibition by various substances present in urine

specimens (12). However, this may further dilute the urine and result in no amplification. Alternatively, urine specimens can be stored at 4^{o}C, instead of -20^{o}C, prior to DNA extraction, as this method has shown to improve PCR amplification of urine specimens (12).

The use of *gyrA* multiplex primers can facilitate real-time analysis of ciprofloxacin susceptibility in *N. gonorrhoeae* and thereby enable informed use of ciprofloxacin to treat susceptible infections. With optimization, these primers can be integrated into a POCT for simultaneous identification of *N. gonorrhoeae* and its ciprofloxacin susceptibility status in less than 2h (if DNA extraction is not required). Such a test, especially with a low cost (i.e. ~$11/sample), could have major implications for resource-poor, high disease burden communities (13, 14). Furthermore, this test could prove useful for re-introducing ciprofloxacin for treatment if susceptibility is indicated. Additionally, this test could help increase surveillance for ciprofloxacin susceptibility, and present coherent findings for surveillance purposes and fill an urgent need, as susceptibility status is not determined with current NAATs. With the proper diagnosis of AMR status and with the use of ciprofloxacin, the use of cephalosporins might be prolonged.

7.2 DIAGNOSIS OF *N. GONORRHOEAE* INFECTIONS

The timely diagnosis of gonorrhea is crucial for proper control of the infection (15). However, an important factor contributing to the rising prevalence of gonorrhea is treatment delay where a patient waits for days for a diagnosis before treatment is initiated (15, 16), and during this time the infection could be passed along to their contacts. The development of POCTs for *N. gonorrhoeae* is recognized as a vital approach to improve timely diagnosis (17-21).

To address this need, a panel of primers unique to *N. gonorrhoeae* was developed and evaluated using DNA from *N. gonorrhoeae* cultures (Chapter 2). Nine of the eleven primer pairs showed 100% sensitivity for *N. gonorrhoeae*. Subsequently, to meet the objective of delivering a diagnostic tool for *N. gonorrhoeae*, these primers were incorporated into the hydrogel platform and assessed using DNA from gonococcal cultures (Chapter 2). Subsequently, the single primer pairs were combined for improved sensitivity with clinical specimens. These newly developed

primer combinations were 100% sensitive with DNA from *N. gonorrhoeae* cultures. Although the primer combinations had lower sensitivities with DNA extracted from remnant Aptima urine specimens (39-64%) and raw urines (0%), promising results were observed with DNA extracted from urine specimens (90-95%). Despite the availability of commercial NAATs with high sensitivities for *N. gonorrhoeae*, the uniqueness of our test is that the results can be provided in real-time and can be a cost-effective tool for resource-constrained settings. If the hydrogel platform can be optimized with these primers, the platform would be a useful tool to introduce as a portable alternative to empirical treatment in resource-limited settings.

7.3 CHARACTERIZATION OF AMR IN *N. GONORRHOEAE* USING REMNANT APTIMA URINE SPECIMENS

NAAT-based *N. gonorrhoeae* diagnosis has resulted in a substantial decline in AMS testing of gonococcal isolates. Thus, it would represent a significant advancement to find a strategy to characterize the AMS status of *N. gonorrhoeae* from positive commercial NAATs (22). Such a strategy would not only allow rational treatment of gonorrhea but would facilitate trend tracking, especially for emerging resistance in gonococci. Thus for my research eight AMR determinants in *N. gonorrhoeae* were assessed using remnant Aptim urine specimens (Chapter 5). These determinants are implicated in resistance to six antibiotics.

With PCR amplification and amplicon sequencing, we observed emerging resistance to cefixime and azithromycin in Saskatchewan. This is highly significant as elevated MICs to azithromycin has prompted the United Kingdom to remove azithromycin from the primary treatment of gonorrhoeae (5). Since azithromycin coupled with ceftriaxone is recommended for gonorrhea treatment in Canada, vigilant and continuous monitoring of emerging resistance is crucial. These findings highlight that valuable information regarding gonococcal AMR can be obtained from characterizing resistant determinants. However, the approach used by us is labour-intensive, time-consuming and costly; therefore a more efficient and user-friendly approach would be valuable for health regions, especially if an isolate's AMS status can be predicted prior to treatment.

7.4 A TRUNCATED TEM-1 AND DELAYED AMPICILLIN HYDROLYSIS

With the worldwide use of ESCs to treat gonococcal infections, a growing concern is that *bla*$_{\text{TEMs}}$ could acquire mutations leading to the evolution of an extended-spectrum β-lactamase (ESBL), which would be able to hydrolyze all ESC antibiotics making gonorrhea an untreatable infection (23-26). We characterized a novel β-lactamase-producing plasmid, pJRD20, from *N. gonorrhoeae* 8903, with an intermediate MIC to penicillin and in which β-lactamase production is significantly delayed (Chapter 6). This finding highlights that although susceptibility to penicillin is not routinely performed, it is useful to monitor penicillinase-producing plasmids of *N. gonorrhoeae*. Characterizing AMR in *N. gonorrhoeae*, especially emerging resistance mechanisms is crucial to managing the spread of AMR globally.

DNA sequencing and structural analyses indicated pJRD20 contains a 6 bp deletion starting at the G of the ATG start codon of *bla*$_{\text{TEM-1}}$ and in turn formed a truncated TEM-1 (TEM-1$_{\text{S-ATG}}$) from a downstream ATG start site. Based on molecular docking studies we proposed the pJRD20 TEM-1 would primarily remain in the cytoplasm, as the N-terminal signal sequence required for periplasmic transportation is absent in this truncated TEM-1. Western blot analysis confirmed these presumptions showing that the resulting protein is a 24 KDa TEM-1 penicillinase, and Mass Spectrometry analysis indicated slow β-lactamase hydrolysis, which supports the hypothesis that the TEM-1 remains in the cytoplasm.

Although *N. gonorrhoeae* 8903 did not confer elevated resistance to penicillin, typical of β-lactamase-producing gonococci, further research is needed to confirm whether the truncated TEM-1 remains in the cytoplasm and is released from gonococci upon cell lysis or uses a novel mechanism to be exported across the cytoplasmic membrane. Our findings highlight the importance of extended monitoring for β-lactamase activity, as these slow β-lactamase producing *N. gonorrhoeae* strains could be easily overlooked. Findings of novel plasmids, such as pJRD20, harbouring *bla*$_{\text{TEM}}$ variants is crucial for the control and management of AMR in *N. gonorrhoeae*.

7.5 CHARACTERIZATION OF AMR DETERMINANTS USING WGS

Whole-genome sequencing has gained widespread popularity as an alternative tool to characterize mutations present in the *N. gonorrhoeae* genome leading to AMR (27-30). In one research study, we demonstrated that WGS based analyses of SNPs in genes attributed to AMR can be a powerful tool to predict gonococcal resistance, in the absence of MIC testing. Furthermore, this research showed that WGS analyses enabled the characterization of strain relatedness and isolate spreading, along with their AMR mechanisms. Although studies have performed WGS using cultures, research is limited in the use of clinical specimens, especially remnant Aptima urine specimens, to characterize AMR in *N. gonorrhoeae*. Therefore, with the use of DNA from Aptima urine specimens and *in silico* generated synthetic datasets, in a different research we showed that a minimum percentage of *N. gonorrhoeae* DNA is required to be present in the sequencing reads to identify an AMR status using WGS. This finding is significant, in that it shows WGS is a powerful tool to extract novel resistance mutations from the *N. gonorrhoeae* genome, however only if a sufficient concentration of nucleic acid is present.

7.6 FUTURE DIRECTIONS

I hypothesized that real-time molecular diagnostic platforms will allow timely diagnosis of *N. gonorrhoeae* and information on its resistance to various antimicrobials used for treatment. Additionally, I proposed that the molecular characterization of resistance determinants would provide a perspective of the spread of AMR and the emergence of resistance in gonococci in local communities. My research supports these presumptions and lays the foundation for preliminary work to develop PCR based diagnostic platforms for *N. gonorrhoeae* and shows emerging AMR trends in Saskatchewan and new AMR determinants.

The goal of my research was to introduce the developed diagnostic tests (i.e. *gyrA* multiplex primers and *N. gonorrhoeae* diagnostic primers) for clinical use. Therefore, to make these tests more user-friendly and rapid, these assays need to be optimized, such that the DNA extraction step could be either automated or not required. To this end, PCR conditions and reagent

concentrations need to be enhanced for each specimen type (i.e. urine, vaginal and urethral swabs) separately. As discussed before, both *gyrA* multiplex primers and *N. gonorrhoeae* identification primers work optimally with DNA from *N. gonorrhoeae* cultures. Therefore, the tests should be optimized to account for low concentrations of DNA in raw urine specimens. To establish this, spiked urine samples could be used at differing concentrations. Alternatively, urine specimens from patients with symptomatic infections (i.e. men are preferred since women tend to have fewer or less prominent symptoms) could be compared to those from patients with asymptomatic (yet Aptima Combo 2 confirmed) gonococcal infections. Asymptomatic 'patients' would ideally be contacts of those who have an Aptima Combo 2 confirmed *N. gonorrhoeae* infection. Once this is established, these tests would be suitable for the introduction into clinical laboratories.

Additionally, the conditions in the hydrogel should be optimized to the primers discussed in this research, such that a portable version of the tests can be evaluated in resource-constrained settings. Once the PCR conditions and specimen concentrations are optimized, these tests could be expanded to assess gonococcal resistance to other antimicrobials. This would allow the creation of an RT-PCR based test panel, where resistance to multiple antimicrobials would be assessed simultaneously and provide important information to clinicians.

The presence of genetic mutations does not always coincide with MIC values indicating resistance, especially when resistance arises through multiple mutations in different genes. Therefore, the prediction of resistance based on molecular analyses should be undertaken with caution. As PCR amplification of single resistance determinants is time-consuming and costly, perhaps the use of WGS may alleviate this problem, as it would provide a comprehensive analysis of the entire genome of the pathogen and would be less expensive and 'rapid' compared to amplicon sequencing. Furthermore, WGS derived results will provide information regarding new and known mutations in genes implicated in AMR and facilitate strain tracking, contact tracing for epidemiological purposes, and the strategic implementation of measures to control the spread of gonococcal AMR.

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APPENDIX A: PERFORMANCE OF *NEISSERIA GONORRHOEAE* SPECIFIC MULTIPLEX DIAGNOSTIC PRIMERS

INTERFACE

This research was carried out using the *N. gonorrhoeae* diagnosis primers discussed in Chapter 2. The primers performed with 100% sensitivity and specificity singly. However, the performance of the single primer pairs was suboptimal with DNA from remnant Aptima urine specimens. In this research, we proposed that by multiplexing the single primer pairs, an improved sensitivity can be achieved with clinical specimens. These new primer combinations were tested first using DNA extracted from *N. gonorrhoeae* cultures and subsequently with DNA from remnant Aptima urine specimens and raw urines. My contribution to this research included conceptualization of the research with guidance from Dr. Dillon, performing laboratory experiments, analyzing data, report writing, and supervising Kristen M Mitzel, who worked on this project as a summer student.

INTRODUCTION

The prevalence of gonorrhea is rising both nationally and globally (1). Treatment costs and associated lost productivity due to the infection are a severe burden to health care systems and economies worldwide (1). Currently, there are no vaccines to prevent *N. gonorrhoeae* infections and diagnosis is mainly based on nucleic acid amplification tests (NAATs) or culture and biochemical characterization of specimens. However, the current costs of commercial NAATs, are beyond the economic capabilities of many resource-limited settings. As such, patients receive empirical treatment. Thus, there is an urgent need to develop an efficient, rapid and cost-effective identification platform for *N. gonorrhoeae* diagnosis. We developed novel primers for the diagnosis of *N. gonorrhoeae* (2; Chapter 2) and have used these primers singly in an RT-PCR platform to identify *N. gonorrhoeae* from DNA extracted from cultures. Our primers showed 100% sensitivity for the identification of *N. gonorrhoeae*. In this research, we explore the utility of these primers in multiplex format to identify gonococcal DNA extracted from cultures, remnant Aptima urine specimens and from raw urines.

MATERIALS AND METHODS

Bacterial Cultures and Clinical Specimens

To determine the sensitivity and specificity of *N. gonorrhoeae* diagnostic primer pairs with clinical specimens, 331 remnant Aptima urine specimens, collectd between 2015 and 2016, were obtained from the Roy Romanow Provincial Laboratory (RRPL), Regina, SK. *N. gonorrhoeae* positive specimens were identified using the Aptima Combo 2 for CT/NG Assay (Hologic Gene-Probe Inc., San Diego CA) at the RRPL. Of the 331 specimens, 301 were *N. gonorrhoeae* positive and 30 were *N. gonorrhoeae* negative. For experiments involving multiplex primer validation, 100 *N. gonorrhoeae* cultures collected from patients in 2018 were obtained from the RRPL. In addition, 20 *N. gonorrhoeae* positive and 20 *N. gonorrhoeae* negative raw urine specimens, collected in 2019, were obtained from the Saskatchewan Health Authority (SHA), Regina region (Regina, SK). These specimens had been tested for *N. gonorrhoeae* with the Aptima Combo 2 assay.

For experiments involving urine specimens, the positive controls consisted of DNA from WHO *N. gonorrhoeae* reference isolates F, P, G, K and N (3), while the negative controls included DNA from *Neisseria weaveri*, *Neisseria wadsworthii*, *Klebsiella oxytoca*, *Staphylococcus aureus* and *Lactobacillus iners* (obtained from the National Microbiology Laboratory (NML), Winnipeg, MB). For validation of multiplex primer combinations, DNA from an additional 15 positive and 15 negative control isolates was used. Here the positive control DNA was extracted from *N. gonorrhoeae* isolates FA19, F62, HongKong-986002, St. Vincent-97606, SK-7461, SK-1902, SK-33414, SK-32318, SK-32420, SK-34638, SK-31352, SK-26539, SK-35426, SK-3542, and SK-31355, from the Dillon culture collection. The negative controls consisted DNA from *Neisseria animaloris*, *Neisseria mucosa*, *Neisseria elongate*, *Neisseria cinereal*, *Neisseria meningitides*, *Neisseria subflava*, *Neisseria polysaccharea*, *Escherichia coli* BL21, *E. coli* V831, *Salmonella* Typhimurium, *Staphylococcus epidermis, Lactobacillus jensenii*, *Lactobacillus gasseri, Moraxella catarrhalis, Enterococcus faecium, Enterococcus faecalis, Pseudomonas aeruginosa,* and pJRD20 (4). DNA from non-*N. gonorrhoeae* isolates were obtained from the NML.

DNA Extraction

This procedure is as discussed in Chapter 4. Briefly, *N. gonorrhoeae* isolates were cultured on GC

medium base (Difco BD Bioscience) supplemented with 1% Kellogg's defined supplement. Plates were incubated at 35-37°C with 5% CO_2 in a humid environment for 18 to 24h (5). DNA extraction from bacterial cultures, remnant Aptima urine specimens, and raw urines was performed using a QIAamp DNA Mini kit (Qiagen) according to the manufacturer's instructions. DNA concentrations from bacterial cultures and remnant Aptima urine specimens were quantified using NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). DNA concentrations from remnant Aptima urine specimens varied significantly (Table A.1). However, the amplification in RT-PCR was unrelated to DNA concentrations. DNA from raw urines were quantified using a Qubit 4 fluorometer (Ther Fisher Scientific) and was normalized to 50ng/µl. Due to variability, DNA concentrations were not standardized for raw urines.

Multiplex Primers

N. gonorrhoeae diagnostic primer pairs were designed as described previously (1; Chapter 2). Primer sequences used in this research are presented in Table A.2. Multiplex primer combinations 1 (P3 and P17-1), 2 (P8-4 and P16), and 5 (P3 and P16) consisted of two different primer pairs, while multiplex primer combinations 3 (P3, P16 and P21-5), 4 (P3, P16 and P31-2) and 7 (P3, P16 and P17-1) consisted of three different primer pairs. Primer combination 6 (P3, P8-4, P16 and P17-1) included four different primer pairs (Table A.3). Targets for all primer pairs are present in multiple copies in the *N. gonorrhoeae* FA1090 genome (NCBI RefSeq NC_002946.2) and in turn, increase the sensitivity of detection (Figure A.1).

Real-Time (RT) PCR

RT-PCR was performed using an Applied Biosystems StepOnePLus instrument (Life Technologies) in a 96-well plate platform (Life Technologies) with SYBR Select Master Mix (Life Technologies). PCR reactions contained 5µl of 2X SYBR-Green, 3µl of combined primer mix (10µM) and 2µl of DNA template (at various concentrations). PCR was initiated with a primary holding and activation at 50°C for 2min, followed by a secondary holding at 95°C for 2 min. PCR was performed for 30 cycles: 95°C for 15 sec and 60°C for 30 sec. The post-PCR melt curve was performed between 65^{o}C to 95^{o}C, with 0.3°C temperature increments for primer combinations 1-5, and 0.1^{o}C temperature increments for primer combinations 6 and 7. Each experiment was performed in duplicate.

Table A.1 DNA concentration variability of remnant Aptima urine specimens.

DNA Concentration	**Number of *N. gonorrhoeae* Positive Specimens (n=301)**	**Number of *N. gonorrhoeae* Negative Specimens (n=30)**
<10 ng/µL	173	25
10-50 ng/µL	128	5
50-100 ng/µL	15	0
>100 ng/µL	10	0

Table A.2 Primer sequences used in this research for detection of *N. gonorrhoeae*, their respective targets and copy numbers.

Primer ID (Product Length)	Sequence (5'->3')[a]	Target Gene[b]	No Targets in Ng FA1090
Primer 3 (139bp) Forward Reverse	 TATGGGGGTTCCTTCGCACC CAGACGGTTGCGGGTTCTTG	NGO05940, NGO06090, NGO06650, NGO1642	4
Primer 8-4 (180bp) Forward Reverse	 TCACGGATGACCGCAGCATA AGACGCTTCACGCCTTCCTT	NGO0773, NGO1200, NGO1703, NGO1137, NGO1164, NGO1262, NGO1641	7
Primer16 (93bp) Forward Reverse	 CGGAACAAGCGTTTTTCAGCG TCTTTGGCTTGTCCGGGTGT	NGO1131, NGO1209	2
Primer 17-1 (73bp) Forward Reverse	 TCCGAAACACGCAAACCGAAA TAGCCCGGGTTGGTATTGCC	NGO1638, NGO0487, NGO1108	3
Primer 21-5 (101bp) Forward Reverse	 GCACGAAACCCGTCCAATCC CAAGACATGCGGCTATGCGG	NGO1085, NGO1652	2
Primer 31-2 (188bp) Forward Reverse	 AAAATCGCGCCGGGTTTGAA AGCTTATCCGCAGCGGTTCT	NGO0480, NGO1113, NGO1631	3

[a]The primer sequences presented in this manuscript are the subject of a United States utility patent (#62/088,332)
[b]Locus Tag ID in the NCBI database

Table A.3 Primer combinations used in this research and primer pairs involved.

Multiplex Primer Combination	Primer Pairs Involved	Melt Curve Temperature
Combination 1	P3 and P17-1	80ºC and 86ºC
Combination 2	P8-4 and P16	83ºC and 88ºC
Combination 3	P3, P16, and P21-5	80ºC, 88ºC, 85.5ºC
Combination 4	P3, P16, and P31-2	80ºC, 88ºC, 83ºC
Combination 5	P3 and P16	80ºC and 88ºC
Combination 6	P3, P8-4, P16 and P17-1	80ºC, 83ºC, 88ºC and 86ºC
Combination 7	P3, P16 and P17-1	80ºC, 88ºC and 86ºC

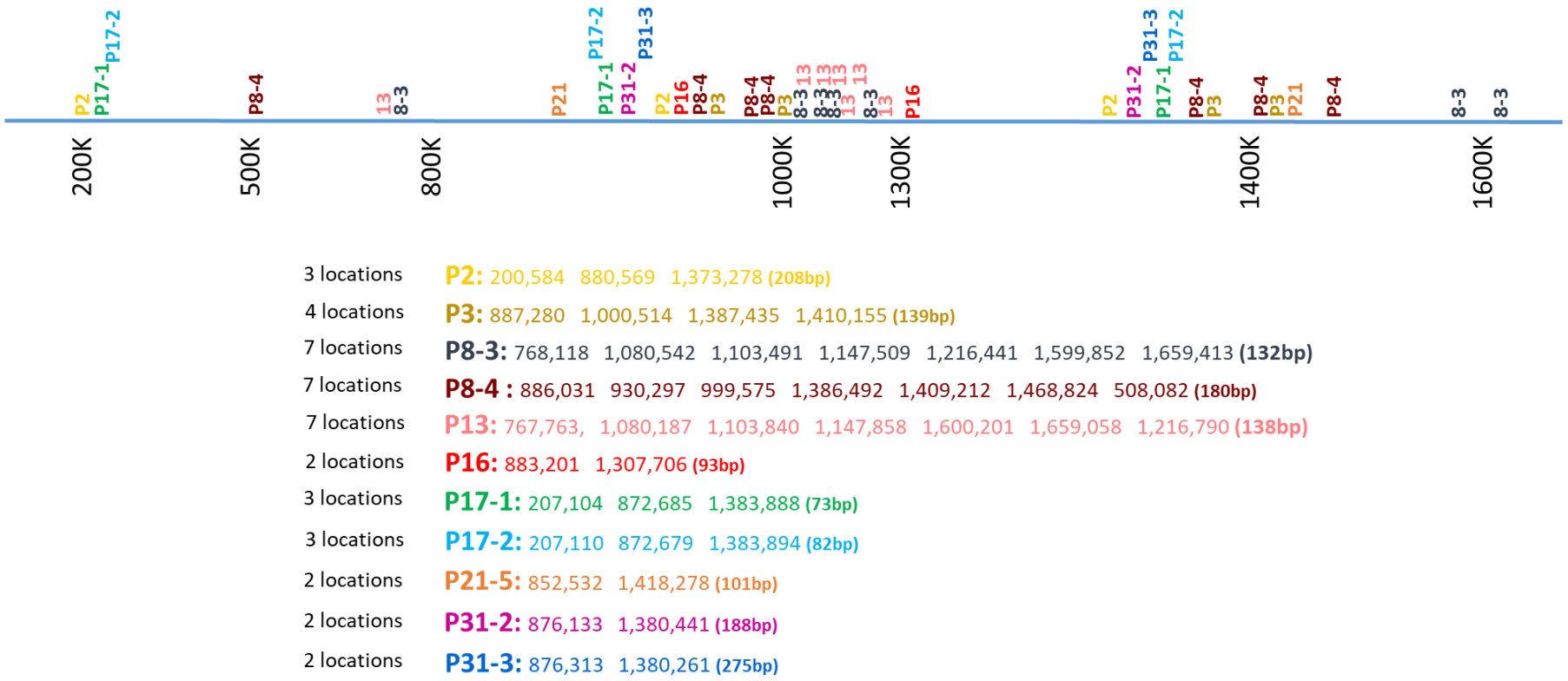

Figure A.1 Representative map of the diagnostic primer binding regions in the *N. gonorrhoeae* FA1090 genome. The starting base pair location of the primer annealing site is indicated beside the primer name (5' to 3') and the PCR product size is indicated within brackets.

RESULTS

Performance of Multiplex *N. gonorrhoeae* Diagnostic Primers

We developed seven different multiplex diagnostic primer combinations from a pool of six different single primer pairs (1; Table A.3). These combinations were created to provide a unique set of melting temperatures for each primer combination. Primer combination 2 was eliminated due to low sensitivity (Table A.4), while the combinations 3 and 6 were eliminated due to overlapping melt curves (Figure A.2). Overlapping melt curves could lead to false-negative identification. Primer combination 4 was eliminated both due to low sensitivity and overlapping melt curves (Figure A.2; Table A.4). The final panel consisted of primer combinations 1, 5 and 7.

These three multiplex primer combinations were further tested with DNA from 100 *N. gonorrhoeae* cultures, and all three combinations had 100% sensitivity (Table A.5). Combination 7 performed the best with having 96% specificity with non-*N. gonorrhoeae* DNA, while combination 1 and 5 exhibited 61% and 74% specificity respectively. Although some non-*N. gonorrhoeae* strains were amplified with these primer combinations (Combination 1 - *N. Animaloris*, *N. weaverii*, *S. epidermis*, *E. coli* BL21, *L. jensenii*, *S. aureus*, *L. iners*, *L. gasseri*, and *E. faecalis*; Combination 5 - *N. Animaloris*, *N. elongate*, *E. coli* V831, *S. aureus*, *M. catarrhalis*, and *L. gasseri*; Combination 7 - *S. aureus*), no non-specific amplification was observed in preliminary tests with single primer pairs (Chapter 2). Similarly, no cross-reactivity was found when single primer pairs were BLAST searched with these non-*N. gonorrhoeae* strains (Chapter 2).

Sensitivity and Specificity of Diagnostic Primer Combinations with Urine Specimens

All three primer combinations (i.e. 1, 5 and 7) had 100% sensitivity and 80% specificity with DNA from positive and negative control isolates (Table A.6). Although 100% specificity was observed previously (1) with single primer pairs, DNA from *S. aureus* was amplified by all primer combinations reducing the specificity. However, when a BLAST search was performed with the primer sequences discussed in this research, cross-reactivity with *S. aureaus* was not observed. A similar search was previously performed for the single primer pairs and was discussed in Chapter 2, Table 2.3.

Table A.4 Sensitivity and specificity of multiplex *N. gonorrhoeae* diagnostic primer combinations with DNA extracted from *N. gonorrhoeae* and non-*N. gonorrhoeae* cultures.

Number of Cultures Tested	**Primer Combination**	**#1**	**#2**	**#3**	**#4**	**#5**	**#6**	**#7**
	Primer pairs	P3, P17-1	P8-4, P16	P3, P16, P21-5	P3, P16, P31-2	P3, P16	P3, P8-4, P16, P17-1	P3, P16, P17-1
N=5	**Sensitivity**	100%	60%	100%	20%	100%	100%	100%
N=10	**Specificity**	90%	100%	100%	100%	90%	95%	95%

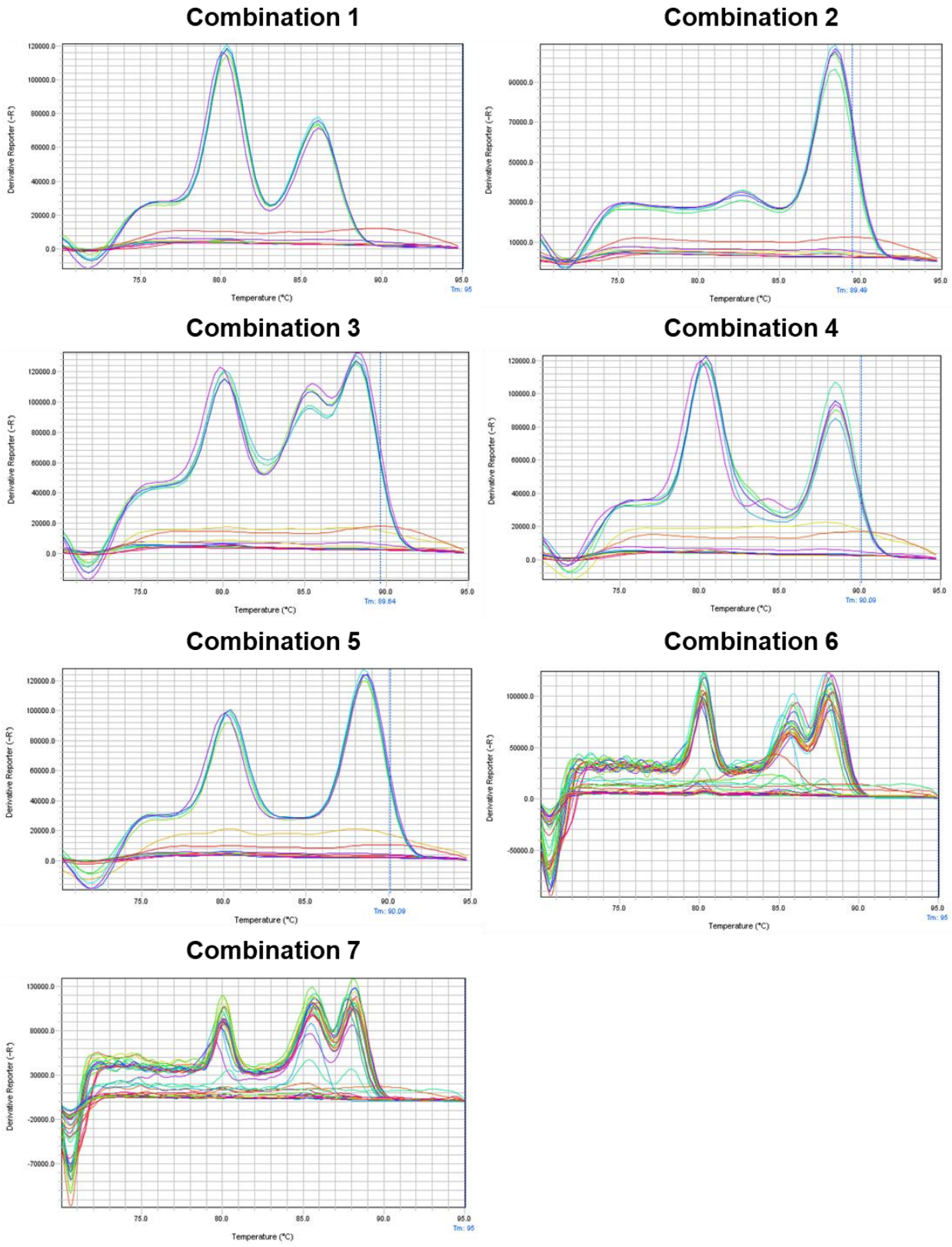

Figure A.2 RT-PCR melt curves for *N. gonorrh*oeae diagnostic primer combinations 1 to 7.

Table A.5 Multiplex *N. gonorrhoeae* diagnostic primer combinations 1, 5 and 7 with DNA from a *N. gonorrhoeae* test panel and control isolates.

	Positive controls (n=20) Sensitivity	**Negative controls (n=23) Specificity**	**DNA from *N. gonorrhoeae* cultures (n=100) Sensitivity**
Primer combo #1	100%	55%[a]	100%
Primer combo #5	95%	70%[b]	100%
Primer combo #7	100%	95%[c]	100%

Non-*Neisseria gonorrhoeae* species amplified: [a]*N. Animaloris*, *N. weaverii*, *S. epidermis*, *E. coli* BL21, *L. jensenii*, *S. aureus*, *L. iners*, *L. gasseri*, and *E. faecalis*; [b]*N. Animaloris*, *N. elongate*, *E. coli* V831, *S. aureus*, *M. catarrhalis*, and *L. gasseri*; [c]*S. aureus*

Table A.6 Sensitivity and specificity of *N. gonorrhoeae* diagnostic primer combinations 1, 5 and 7 with DNA extracted from urines and raw urine specimens.

	Primer Combination		
	#1	**#5**	**#7**
	Sensitivity		
DNA from *N. gonorrhoeae* cultures (n=5)	100%	100%	100%
DNA from *N. gonorrhoeae* positive urines (n=20)	90%	95%	95%
N. gonorrhoeae positive raw urines (n=20)	0%	0%	0%
	Specificity		
DNA from non-*N. gonorrhoeae* cultures (n=5)	80%	80%	80%
DNA from *N. gonorrhoeae* negative urines (n=20)	25%	35%	25%
N. gonorrhoeae negative raw urines (n=20)	100%	100%	100%

Primer combination 1 had 90% sensitivity while the primer combinations 5 and 7 each had 95% sensitivity with DNA from *N. gonorrhoeae* positive urines (Table A.6). None of the primer combinations was able to amplify directly from *N. gonorrhoeae* positive raw urines (i.e. urines with no DNA extraction step). Primer combinations 1 and 7 had 25% specificity with DNA from *N. gonorrhoeae* negative urines, while the primer combination 5 had 35% specificity. All three primer combinations had 100% specificity with *N. gonorrhoeae* negative raw urines.

As discussed above, the *N. gonorrhoeae* diagnostic primer combinations had 100% sensitivity with DNA from cultures and demonstrated high sensitivities with DNA extracted from urines. Thus in this research, we utilized the diagnostic primer combinations 1, 5 and 7 to test the primers with DNA from Aptima urine specimens. For this, DNA from *N. gonorrhoeae* positive (n=36) and negative (n=36) remnant Aptima urine specimens was tested (Table A.7). A 94% specificity was observed for all three primer combinations. Although relatively low, primer combination 5 had the highest sensitivity at 64%. Primer combination 1 had 61% and combination 7 had 39% sensitivity respectively. Due to the low sensitivity, these results were indicative that DNA from remnant Aptima urine specimens was not ideal for *N. gonorrhoeae* diagnosis. This finding supported our previous observations where we noticed low amplification success both with PCR (Chapter 5) and with whole-genome sequencing (Appendix C) approaches using DNA from remnant Aptima urine specimens.

Table A.7 Performance of *N. gonorrhoeae* diagnostic primer combinations 1, 5 and 7 with DNA from remnant Aptima urine specimens.

Primer Combination	***N. gonorrhoeae* positive specimens (n=36) Sensitivity**	***N. gonorrhoeae* negative specimens (n=36) Specificity**
#1	61%	94%
#5	64%	94%
#7	39%	94%

DISCUSSION

The *N. gonorrhoeae* diagnostic primer combinations have high sensitivities and specificities with DNA extracted from *N. gonorrhoeae* cultures and urine specimens. This is promising, as the current costs associated with culturing and training of laboratory personnel, as well as the cost of commercial NAATs, are beyond the economic capability of many resource-limited settings. The extremely low sensitivity (0%) observed with *N. gonorrhoeae* negative raw urine specimens (Table A.6) may be attributed to relatively low concentrations of overall DNA in the specimen, and this may in turn account for the extremely high specificity (i.e. 100%) with positive urine specimens. As such, the use of DNA extracted from urines may be a better alternative to using raw urines (i.e. urines without DNA extraction) in RT-PCR based diagnostic assays. However, it is important to note that we have only tested a limited number of urine specimens and have not tested other types of clinical specimens such as cervical, urethral, pharyngeal and rectal swabs, which may prove more effective without a DNA extraction step. However, such specimens first need to be evaluated with these primer combinations. Despite the limitations, our findings show promise in using the multiplex *N. gonorrhoeae* diagnostic primers and DNA extracted from urine specimens to identify *N. gonorrhoeae*. Thus, the RT-PCR based platform discussed in our work can be a rapid, user-friendly and more affordable alternative for the more expensive commercial NAATs for *N. gonorrhoeae* diagnosis.

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APPENDIX B: PRELIMINARY ANALYSIS OF *NEISSERIA GONORRHOEAE* STRAINS FROM A LOCAL "OUTBREAK"

INTERFACE

This research was conducted to characterize known antimicrobial resistance (AMR) determinants from a collection of *N. gonorrhoeae* strains identified as being similar using several different typing schemes. DNA from twenty strains were sent for whole-genome sequencing (WGS). However, only five strains remained viable. Therefore, only the results from PCR amplification with NG-STAR primers for these five strains are presented in this appendix. My contribution to this research included DNA extraction from gonococcal cultures, analysis of amplicon sequencing reads, and supervision of the technician (Jin Wang) working on the research. WGS reads were subsequently analyzed by Dr. Reema Singh. The manuscript for this research is in preparation.

MATERIALS AND METHODS

Bacterial Cultures and DNA Extraction

Five different *N. gonorrhoeae* isolates from two different outbreaks were used in this research (isolates 3644, 3648, 5707, and 6525 from outbreak 1, and isolate 883511 from outbreak 2; Dillon Culture Collection). *N. gonorrhoeae* isolates were stored at -80°C in brain heart infusion medium (Difco BD Bioscience) with 20% glycerol. Aliquots from frozen inoculum were cultured on GC medium base (Difco BD Bioscience) supplemented with 1% Kellogg's defined supplement. Plates were incubated at 35-37°C with 5% CO_2 in a humid environment for 18 to 24h (1). Cells were collected from agar surfaces with a sterile loop and DNA extraction was performed using a QIAamp DNA Mini kit (Qiagen) according to the manufacturer's instructions. DNA was quantified using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific).

PCR Amplification and DNA Sequencing

DNA was PCR amplified using *Taq* polymerase and primer pairs recommended by the *N. gonorrhoeae* Strain Typing for Antimicrobial Resistance (NG-STAR) typing scheme (https://ngstar.canada.ca/alleles/query?lang=en; Table B.1). Primer sequences were synthesized at

Thermo Fisher Scientific. Amplicons were confirmed for the correct size by agarose gel electrophoresis and were sequenced in both forward and reverse directions by Sanger sequencing (Eurofins Genomics, Louisville KY, USA). Point mutations in *penA* (M32091.1), *mtrR* (Z25796.1), *porB1b* (M21289.1), *ponA* (AB727718.1), *gyrA* (U08817.1), *parC* (U08907.1) and 23S rRNA (X67293.1) were determined using Clone Manager 8 Professional (Scientific & Educational Software, NC, USA).

RESULTS

Seven different genes implicated in AMR in *N. gonorrhoeae* were characterized (Table B.2). All five isolates carried 345D and F540L substitutions in PenA and a C2605T nucleotide substitution in the 23S rRNA. *N. gonorrhoeae* 883511 had an additional C2599T substitution in the 23S rRNA domain. The L421P substitution in PonA was only observed in three isolates. An A- deletion in the promoter region of *mtrR* and G101K, G120K and A121D substitutions in PorB1b were also observed in the same three isolates with the PonA substitution (*N. gonorrhoeae* 3644, 3648 and 883511). *N. gonorrhoeae* 5707 and 6525 isolates only contained G101K substitution in PorB1b and did not harbour the A- deletion in the *mtrR* promoter region. Only *N. gonorrhoeae* 5707 carried a D86N substitution in ParC and none of the isolates carried GyrA substitutions.

Table B.1 List of primers used to amplify *N. gonorrhoeae* AMR markers (2).

AMR marker	Primer name	Primer sequence (5' to 3')	Annealing Temperature	Product size (bp)
penA	PenA-A1(forward) PenA-B1(reverse)	CGGGCAATACCTTTATGGTGGAAC AACCTTCCTGACCTTTGCCGTC	62°C	669
	PenA-A2(forward) PenA-B2 (reverse)	AAAACGCCATTACCCGATGGG TAATGCCGCGCACATCCAAAG	55°C	581
	PenA-A3(forward) PenA-B3 (reverse)	GCCGTAACCGATATGATCGA CGTTGATACTCGGATTAAGACG	58°C	863
mtrR	MTR1(forward) MTR2 (reverse)	AACAGGCATTCTTATTTCAG TTAGAAGAATGCTTTGTGTC	58°C	916
porB1b	por-NGMAST-F (forward) por-NGMAST-R (reverse)	CAAGAAGACCTCGGCAA CCGACAACCACTTGGT	58°C	737
ponA	ponA1-f (forward) ponA1-r (reverse)	CGCGGTGCGGAAAACTATATCGAT AGCCCGGATCGGTTACCATACGTT	65°C	1240
gyrA	GYRA-1(forward) GYRA-2 (reverse)	AACCCTGCCCGTCAGCCTTGA GGACGAGCCGTTGACGAGCAG	68°C	270
parC	parC F (forward) parC R (reverse)	GTTTCAGACGGCCAAAAGCC GGCATAAAATCCACCGTCCCC	62°C	332
23S rRNA	gonrRNAF (forward) gonrRNAR2 (reverse)	ACGAATGGCGTAACGATGGCCACA TTCGTCCACTCCGGTCCTCTCGTA	55°C	712

Table B.2 Characterization of point mutations in known AMR determinants in *N. gonorrhoeae* using NG-STAR primer pairs.

Isolate ID	PenA	MtrR	PorB1b	PonA	GyrA	ParC	23S rRNA	MIC[c] Range
3644[a]	345D F504L	A-	G101K G120K A121D	L421P	WT	WT	C2605T	Penicillin: 0.25-256 mg/L Tetracycline: 0.25-16 mg/L Erythromycin: ≤0.063-0.125 mg/L Spectinomycin: 16-32 mg/L Ceftriaxone: 0.001-0.004 mg/L
3648[a]	345D F504L	A-	G101K G120K A121D	L421P	WT	WT	C2605T	
5707[a]	345D F504L	WT	G101K	WT	WT	D86N	C2605T	
6525[a]	345D F504L	WT	G101K	WT	WT	WT	C2605T	
883511[b]	345D F504L	A-	G101K G120K A121D	L421P	WT	WT	C2599T C2605T	Penicillin: 2-4 mg/L Tetracycline: 2-4 mg/L Erythromycin: 2-4 mg/L Spectinomycin: 16-32 mg/L Ceftriaxone: 0.016-0.032 mg/L

[a]Isolates from outbreak 1
[b]Isolates from outbreak 2
[c]Minimum inhibitory concentrations. MICs were reported as a range for all the isolates from the outbreak.

DISCUSSION

Specific mutations in *penA*, *mtrR*, *porB*, and *ponA* are indicative of resistance to penicillin, while *mtrR* and *porB* mutations are indicative of reduced susceptibility to tetracycline. Thus based on our analysis, all five isolates are chromosomally resistant to penicillin and isolates 3644, 3648 and 883511 have low-level chromosomal resistance to tetracycline. Although substitutions in RpsJ are associated with high-level tetracycline resistance, *rpsJ* was not characterized in this research. Isolates 5707 and 6525 have wild-type *mtrR* promoter sequences and a G101K substitution in PorB1b. Therefore these isolates are intermediately susceptible to penicillin and likely susceptible to tetracycline. As the ParC substitutions alone do not confer ciprofloxacin resistance, all five isolates are deemed ciprofloxacin susceptible. Mutations in 23S rRNA are indicative of reduced susceptibility to azithromycin. Therefore, all five isolates could be predicted to have elevated MICs to azithromycin. However, it is important to note as only one of the four alleles of the peptidyl-transferase loop region in domain V of 23S rRNA was sequenced with these primers, the identification of elevated MICs to azithromycin is presumptive. Additionally, all five isolates are susceptible to cefixime and ceftriaxone. The predicted susceptibilities are in agreement with the reported MIC ranges for these isolates. However, since MICs were reported as a range for all the isolates from the outbreak, it was difficult to do a direct comparison between predicted susceptibility and observed MICs for a given isolate.

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APPENDIX C: IS WHOLE GENOME SEQUENCING AN EFFECTIVE TECHNIQUE FOR THE IDENTIFICATION AND CHARACTERIZATION OF *NEISSERIA GONORRHOEAE* IN APTIMA URINE SAMPLES?

INTERFACE

With the use of DNA from Aptima urine specimens and *in-silico* generated synthetic datasets, this research shows that a minimum percentage of *N. gonorrhoeae* DNA to be present in the sequencing reads is critical, to ascertain an isolate's antimicrobial susceptibility (AMS) status using whole-genome sequencing (WGS). My contribution to this preliminary research included extracting DNA from remnant Aptima urine specimens. Bioinformatics analyses were performed by Dr. Reema Singh. The manuscript for this research is in preparation.

ABSTRACT

WGS as a molecular diagnostics tool is gaining widespread interest in clinical microbiology. However, this technique is not always directly applicable to clinical specimens due to various limitations. This research evaluated the performance of bioinformatics analysis of *N. gonorrhoeae* WGS data from 99 artificial datasets and 9 remnant Aptima urine specimens. The 99 artificial datasets were generated by mixing *N.gonorrhoeae* and human WGS read sets using the "Reservoir Sampling" algorithm. Using simulated data, it was found that when the amount of human reads in the sample increase, the average percentage of the GC content of each sample decreases, while the total number of contigs per assembly increases. Synthetic dataset results were verified by analyzing nine remnant Aptima urine specimens. We showed that at least 5% of reads from *N. gonorrhoeae* should be present in the total WGS read datasets to determine an isolate's antimicrobial susceptibility pattern.

INTRODUCTION

Although nucleic acid amplification tests (NAATs) are widely used to confirm gonorrhea infections, an isolate's antimicrobial susceptibility (AMS) status (1-3) is not predicted using current NAATs. To address this limitation, WGS, especially using gonococcal cultures, has gained widespread popularity (3-7). However, despite the use of WGS techniques for strain typing and characterization of resistance determinants from *N. gonorrhoeae* (6-8), research is limited in the use of WGS from *N. gonorrhoeae* positive clinical specimens, such as remnant Aptima urine specimens, for characterizing gonococcal AMS status. The main limitation is that the extraction of DNA from remnant Aptima specimens (i.e. remaining specimen following *N. gonorrhoeae* identification with Aptima Combo 2 assay) presents significant challenges. For example, Aptima buffer is a transportation medium that lyses all cells (human and bacterial) in the specimen, making it extremely difficult to enrich *N. gonorrhoeae* and other bacterial DNA (9, 10). Nonetheless, several studies have utilized remnant Aptima urine specimens for PCR amplification of known AMR determinants (2, 11, 12). In the present research, we evaluated the performance of bioinformatics analysis on WGS reads from 99 *in-silico* generated datasets and 9 remnant Aptima urine specimens.

MATERIALS AND METHODS

Artificial Dataset Generation

Synthetic datasets were prepared by mixing *N. gonorrhoeae* and human WGS read datasets at several (10%-90%) combinations. The WGS paired-end datasets generated using MiSeq platforms (600 chemistry used for sequencing) were downloaded from the NCBI SRA repository (13; Table C.1). To make Dataset-1, 10% (35,976) of the total reads (sample no 27931), the same set of pair-end reads were replaced with 35,976 paired-end reads from a human WGS dataset (sample no ERR3181737). A random set of read-pairs were extracted by using "seqtk sample" (https://github.com/lh3/seqtk) command from *N. gonorrhoeae* and human read sets. The algorithm used for random sampling is "Reservoir Sampling". Similarly, Datset-2 was created by replacing 20% (71,952) of the total reads with 71,952 paired-end reads from a human WGS dataset using "seqtk random sampling". Various combinations of synthetic datasets generated using Pair1 are

shown in Table C.2. Similarly, a total of 99 different combinations were generated using dataset pairs given in Table C.1. Synthetic datasets were generated using *in-house* Perl scripts. *De-novo* assemblies were generated from each dataset by using SPAdes (14) v 3.10.1 (--k 21, 33, 55, 77, 99, 127, --pe1-1, -- pe1-2, --careful and –cov-cutoff auto) implemented in Gen2Epi (15). Resulting contigs were searched for AMR genes.

DNA Extraction and Whole Genome sequencing

DNA from nine remnant Aptima urine specimens was extracted using QIAamp DNA Mini kit (Qiagen) according to manufacturer instructions. The DNA concentration was measured using NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) and 5ng/µl of DNA from each isolate was used for whole genome sequencing (The Donnelley Sequencing Center, University of Toronto, Toronto ON, Canada). Paired-end reads (35-301 bp in length) were generated on the Illumina Miseq platform using the Nextera XT library preparation kit with 100X coverage.

Bioinformatics Analysis

Raw reads were trimmed using Trimmomatic v 0.36 (16) with a Phred quality score of < 20. *De-novo* assemblies were obtained using Spades v 3.10.1 (parameters "--k 21, 33, 55, 77, 99, 127, --pe1-1, -- pe1-2, --careful and –cov-cutoff auto") (12). Contamination with human DNA and DNA from urogenital bacterial species are the main problems in samples collected from patients with sexually transmitted infections. Therefore, taxonomic labels were assigned to raw reads using Kraken v 1.1 (17). Kraken libraries for bacteria, archaea, fungi, human, plasmid, protozoa, and virus were used to create a database for read binning. Read binning results were confirmed by aligning the raw reads to the reference *N. gonorrhoeae* FA1090 genome (NC_002946.2). Assembled contigs were further searched for sequences of known antimicrobial resistant determinants: *porB* (Z69259.1), *porB1b* (EU719207.1), *tbpB* (U65219.1), *penA* (M32091.1), *mtrR* (KT954119.1), *ponA* (AB727718.1), *gyrA* (U08817.1), *parC* (U08907.1), and 23S rRNA (X67293.1) downloaded from the NCBI nucleotide database (https://www.ncbi.nlm.nih.gov/nucleotide).

Table C.1 *N. gonorrhoeae* and Human WGS datasets used in this research.

***N. gonorrhoeae* Sample**	**Total Reads**	**Read Size (bp)**	**Human**	**Total Reads**	**Read Size (bp)**	**Combination[a]**
27931	359,760	39-301	ERR3181737	535,527	35-250	Combination1
28861	581,813	35-251	ERR3181738	726,987	35-250	Combination2
33679	504,310	35-251	ERR3181740	606,080	35-250	Combination3
34106	458,761	35-251	ERR3181730	721,803	35-250	Combination4
34108	733,738	35-251	ERR3181731	756,895	35-250	Combination5
34442	718,889	89-301	ERR3181714	878,519	35-250	Combination6
34688	664,878	72-301	ERR3181719	744,968	35-250	Combination7
35476	611,542	35-301	ERR3181732	953,472	35-250	Combination8
36090	567,537	127-301	ERR3181723	817,848	35-250	Combination9

[a]Each combination has 11 samples

Table C.2 Bioinformatics analysis of *N. gonorrhoeae* datasets.

***N. gonorrhoeae* Sample**	**% GC**	**Total contigs**
27931	52.71	89
28861	52.44	110
33679	52.53	147
34106	52.54	179
34108	52.53	159
34442	52.51	112
34688	52.49	108
35476	52.68	110
36090	52.5	128

RESULTS

Synthetic Datasets

A total of 99 artificial datasets were generated from different *N. gonorrhoeae* and human read set combinations (Table C.1). First, to identify how *in silico* generated datasets differ from *in vitro* generated *N. gonorrhoeae* reads, we compared the average GC content of all 99 synthetic WGS datasets. The average GC content of *N. gonorrhoeae* and the human genome is 52.4% and 39%, respectively. As shown in Figure C.1, the average GC percentage drops 38% as the number of human-read sets increases in each dataset. Second, the distribution of the total number of assembled contigs is shown in Figure C.2. The assemblies vary in size ranging from the smallest contigs of 128 bp to the largest of 278,679 bp. We observed that the total number of contigs increase per assembly with the increase of human reads in each dataset. However, to measure the completeness and authenticity, these contigs generated from human contaminated reads require additional analysis such as annotation and quality check.

Finally, we observed that all AMR determinants (*porB, porB1b, tbpB, penA, mtrR, ponA, gyrA, parC,* and 23S rRNA) were present in the contig assemblies generated from all 99 artificial datasets. The only exception was the artificial dataset 11, with 1% *N. gonorrhoeae* reads, where most of the AMR determinant is either missing or partially present. For example, AMR determinants missing in data combinations include; *porB* and *porB1b* in combination 6, *mtrR* and *parC* in combination 4, *tbpB* in combination 3, *gyrA* in combination 2, and *penA* and *ponA* in combination 1. In combinations, 7, 8 and 9 artificial datasets assembled all nine AMR determinants.

Validation of synthetic datasets results

To validate the results obtained from synthetic WGS dataset analysis, we performed WGS analysis on *N. gonorrhoeae* positive nine remnant Aptima urine specimens. The detailed bioinformatics analysis results are shown in Table C.3. Read binning results provided a thorough insight into the overall organism distribution in remnant Aptima urine specimens (Table C.4). Here we

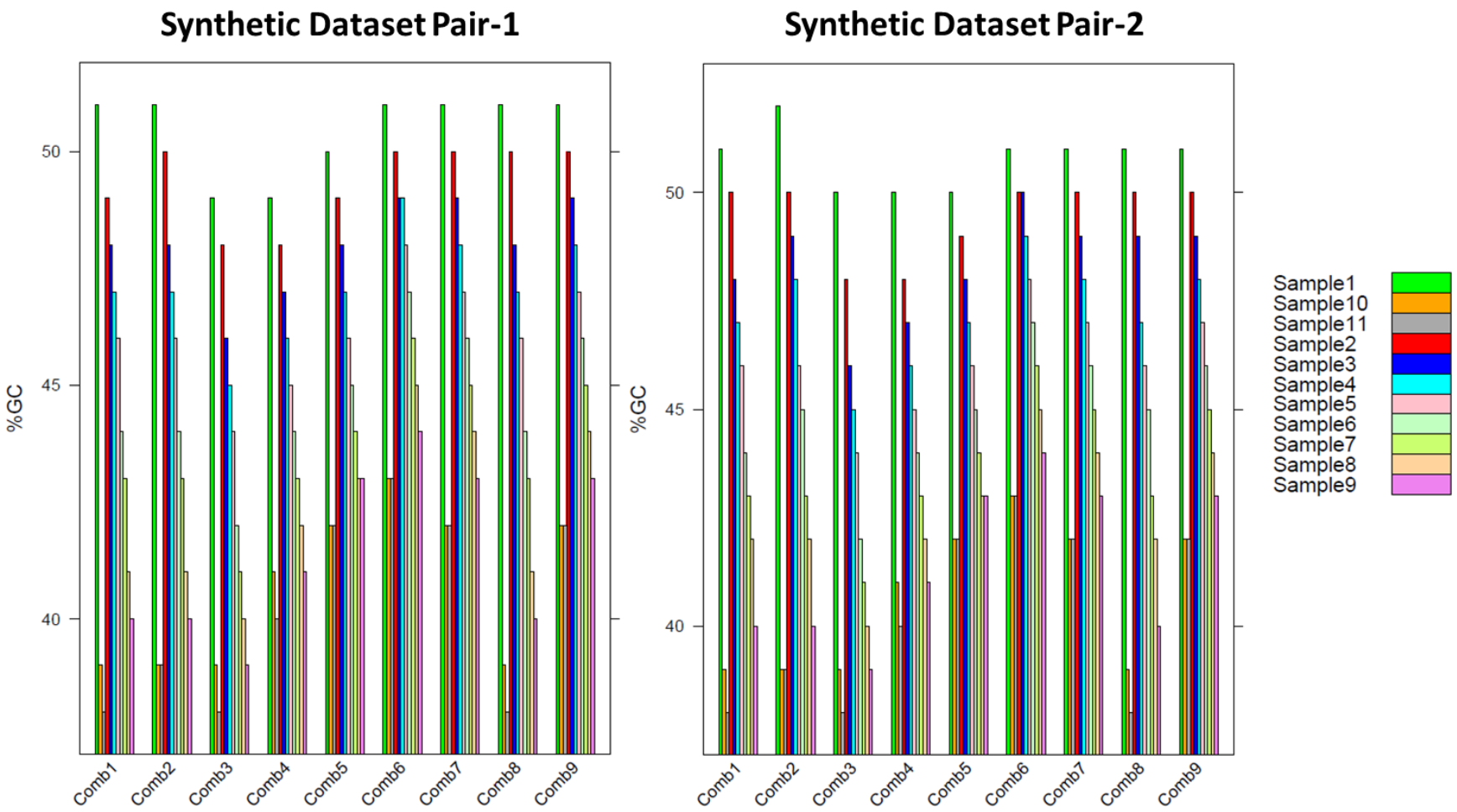

Figure C.1 Comparison between 99 synthetic datasets based on average % GC.

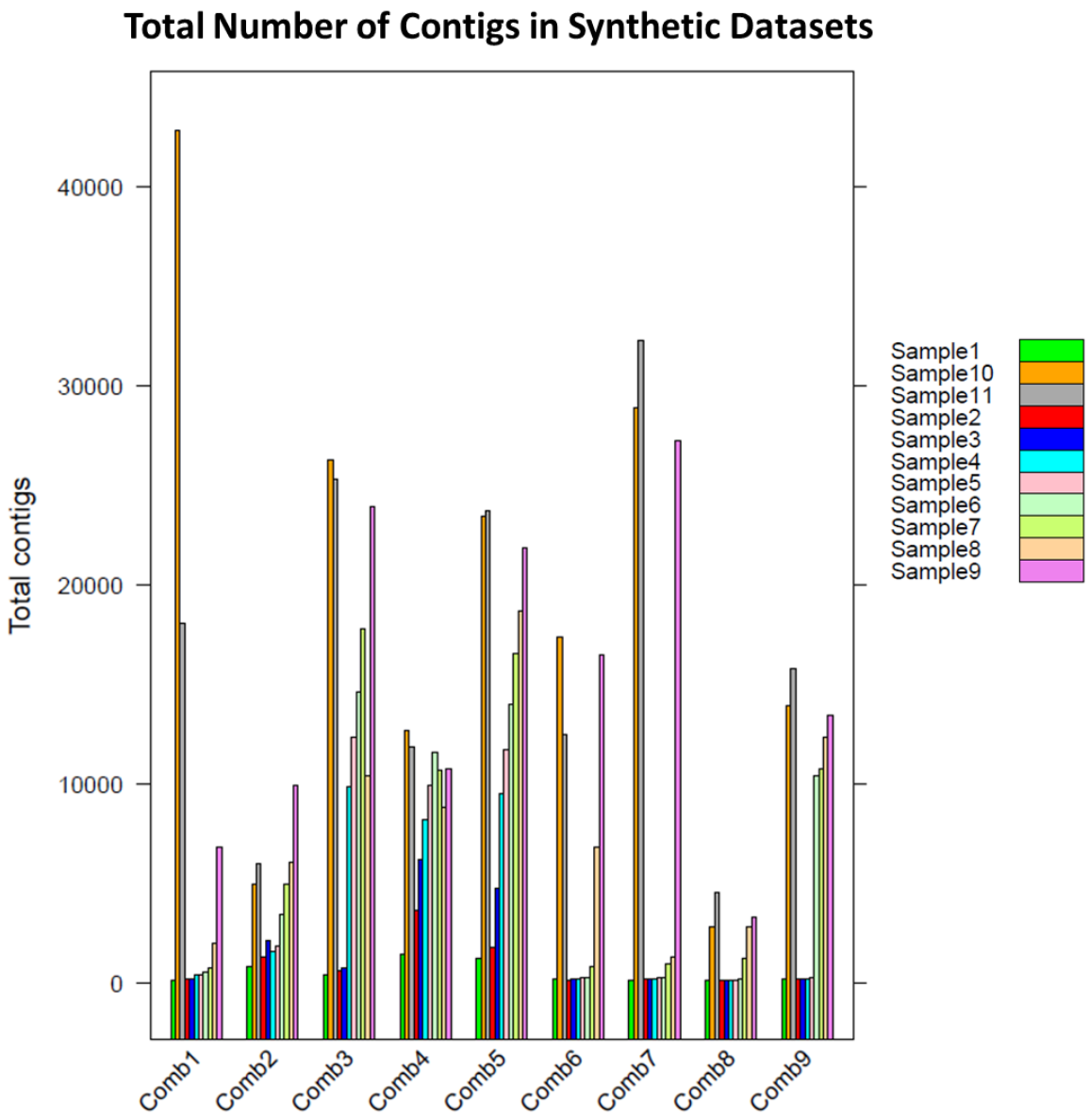

Figure C.2 Comparison between 99 synthetic datasets based on the total number of assembled contigs.

Table C.3 Summary of bioinformatics analysis of WGS reads from nine *N. gonorrhoeae* positive remnant Aptima urine specimens.

Samples	**Total Reads**	**Alignment to *N. gonorrhoeae* FA1090 genome**	**Number of contigs**	
			Raw Reads	**Trimmed Reads**
U205	1,272,329	0.16%	28,572	272
U215	1,581,119	0.21%	864	207
U238	1,308,487	0.09%	1,045	336
U244	1,431,331	0.22%	25,179	186
U281	1,460,481	0.11%	1,140	243
U283	1,174,304	0.11%	27,601	274
U35	1,406,793	0.14%	861	218
U2	1,142,161	0.11%	18,365	204
U30	1,905,260	0.07%	1,214	332

Table C.4 Read binning results of WGS read sets from nine remnant Aptima urine specimens.

Samples	**Human Reads**	***N. gonorrhoeae* Reads**	**Other Species (Number of reads)**
U205	1,267,783 (99.64%)	2,639	*N. lactamica* (2), *N.meningitidis* (16), *Neisseria*_sp_KEM232 (2), Human polyomavirus (20)
U215	1,572,138 (99.43%)	3,707	*N. lactamica* (4), *N. meningitidis* (19), *N.sicca* (2), *Cutibacterium acnes* (18), *Sphingobium yanoikuyae* (12)
U238	1,304,818 (99.72%)	1,617	*N. meningitidis* (5), *Cutibacterium acnes* (30), *Escherichia coli* (25)
U244	1,423,503 (99.45%)	5,137	*N. meningitidis* (17), *Cutibacterium acnes* (9)
U281	1,422,629 (97.41%)	2,200	*N. meningitidis* (10), *Mycoplasma hominis* (286), *Cutibacterium acnes* (54), *Gardnerella vaginalis* (6283), *Mobiluncus curtisii* (86), *Mageeibacillus indolicus* (102), *Porphyromonas gingivalis* (75), *Porphyromonas asaccharolytica* (242), *Prevotella ruminicola* (26), *Prevotella denticola* (86), *Prevotella intermedia* (1277), *Prevotella melaninogenica* (136), *Prevotella dentalis* (55), *Prevotella enoeca* (98), *Prevotella fusca* (125), *Prevotella scopos* (185), *Prevotella_sp_oral_taxon_299* (216), *Sneathia amnii* (3396), *Fusobacterium nucleatum* (294), *Pseudomonas fluorescens* (32)
U283	1,170,719 (99.69%)	1,821	*N. meningitidis* (14), *Cutibacterium acnes* (24), Human polyomavirus (10)
U35	1,401,771 (99.64%)	2,751	*N. meningitidis* (17), *Cutibacterium acnes* (12)
U2	1,138,707 (99.70%)	1,657	*N. meningitidis* (12), *Cutibacterium acnes* (14), *Klebsiella pneumoniae* (17), *Klebsiella variicola* (22)
U30	1,897,433 (99.59%)	1,832	*N. meningitidis* (6), *Gardnerella vaginalis* (1539), *Cutibacterium acnes* (35), *Lactobacillus crispatus* (52), *Lactobacillus jensenii* (54), *Pseudomonas fluorescens* (16)

identified other bacterial species such as *Gardnerella vaginalis*, *Prevotella intermedia*, and *Sneathia amnii* along with several other low abundant species in 2/9 specimens. A large number of reads (99%) belonged to the human, while only <1% reads resulted from *N.gonorrhoeae*, and as such is insufficient to characterize the pathogen (Table C.3).

DISCUSSION

We evaluated the performance of WGS on clinical specimens using bioinformatics analysis of remnant Aptima urine specimens and found the minimum amount of DNA required to identify *N.gonorrhoeae* from such specimens. We first analyzed 99 *in-silico* generated datasets by mixing human and *N.gonorrhoeae* WGS read datasets at different percentages. Subsequently, WGS data from nine remnant Aptima urine specimens was analyzed to reveal that only <1% *N. gonorrhoeae* reads were present following WGS of these specimens. This was indicative that a threshold quantity of *N. gonorrhoeae* DNA in Aptima specimens is key to the efficient use of WGS on these specimens. This finding is in agreement with two previous studies (18, 19) that describe the limitations of extracting DNA for WGS from remnant Aptima specimens in the case of *N. gonorrhoeae* and *C. trachomatis*.

The other key concern when performing WGS from remnant Aptima specimens is the presence of reads from other microbial species and contamination with host (i.e. human) DNA (20). A large number of human reads (i.e. 99%) in all 9 remnant Aptima specimens indicate the need to develop more efficient methods to extract human DNA prior to genome sequencing. The screening of AMR determinants against assembled contigs indicated that at least 5% reads from *N. gonorrhoeae* should be present in the WGS data to determine an isolate's susceptibility profile when using remnant Aptima urine specimen. Further studies where WGS data is obtained from other types of clinical specimens (i.e. urines, swabs) are needed to validate our findings.

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APPENDIX D: OTHER MANUSCRIPTS NOT DISCUSSED IN THE THESIS

Manuscripts under Review

1. Parma NR, Singh R, Martin I, Perera SR, Demczuk W, Kusalik A, Minion J, and Dillon JR. Whole Genome Sequencing of *Neisseria gonorrhoeae* for Simultaneous Prediction of Antimicrobial Resistance, Genotypes, and Transmission Linkages. *Submitted on December 16th 2019 to Antimicrobial Agents and Chemotherapy*: AAC02514-19. Under revision.

Manuscripts in Preparation

1. Vidovic S, Singh R, Li H, Vujanovic S, Liao M, Perera SR, and Dillon JR. Tracking *Neisseria gonorrhoeae* Epidemics: How Well have Typing Schemes Worked. *Submitting to the Journal of Clinical Microbiology*.