Molecular epidemiology and antimicrobial resistance of *Neisseria gonorrhoeae* in men at risk in Gauteng, South Africa

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Molecular epidemiology and antimicrobial resistance of *Neisseria gonorrhoeae* in men at risk in Gauteng, South Africa

by

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DECLARATION

I, the undersigned, declare that the thesis, which I hereby submit for the degree PhD (Medical Microbiology) at the University of Pretoria, is my own original work and has not been previously submitted by me for a degree at this or any other tertiary institution. I further declare that all sources cited or quoted are specified and recognised by means of an inclusive list of references.

_____ _____

Signature Date

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AMR	Antimicrobial resistance
AST	Antimicrobial susceptibility testing
ATCC	American Type Culture Collection
BASHH	British Association for Sexual Health and HIV
CDC	Centres for Disease Control and Prevention
CHC	Community health centre
CI	Confidence interval
CSW	Commercial sex worker
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
DHPS	Dihydropteroate synthetase
ECOFF	Epidemiological cut-off
EUCAST	The European Committee on Antimicrobial Susceptibility Testing
FRET	Fluorescence resonance energy transfer
FVU	First void urine
GARDP	Global Antibiotic Research and Development Partnership
GASP	Global gonococcal antimicrobial surveillance program
GP	General practitioner
HIV	Human immunodeficiency virus
IM	Intramuscular

MALDI-TOF	Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry
MATE	Multidrug toxic compound extrusion
MDR	Multi-drug resistant
MFS	Major facilitator family
MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing
MSM	Men who have Sex with Men
MUS	Male urethritis syndrome
NAATS	Nucleic acid amplification tests
NG-MAST	*Neisseria gonorrhoeae* Multi-antigen sequence typing
NG-STAR	*Neisseria gonorrhoeae* sequence typing for antimicrobial resistance
NYC	New York City agar
PBP	Penicillinase binding protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PHC	Primary healthcare
PO	Per os
POC	Point of care
QRDR	Quinolone resistance determining regions
RCT	Randomised controlled trial
RGT	Resistance guided therapy

RNA	Ribonucleic acid
RND	Resistance nodulation
SNP	Single nucleotide polymorphism
ST	Sequence type
STI	Sexually transmitted infection
STR	Short Tandem Repeats
TEM	Temoniera
Tm	Melting temperatures
TOC	Test of cure
UK	United Kingdom
UN	United Nations
US	United States
WGS	Whole genome sequencing
WHO	World Health Organization
XDR	Extensively drug resistant

LIST OF PUBLICATIONS, CONFERENCE ATTENDANCE AND AWARDS

Publications

1. **Maduna, L.D**., Kock, M.M., Medina-Marino, A., Klausner, J.D., Peters, R.P.H. 2019. Impact of specimen storage temperature and time on the implementation of Genexpert® testing for sexually transmitted infections in resource-constraint settings. *Journal of Microbiological Methods*. 165 (2019) 105719.

2. **Maduna, L.D**., Laumen, J.G., Radebe, O., Kock, M.M., Peters, R.P.H 2019. Failure of syndromic management due to drug-resistant *Mycoplasma genitalium* infection in South Africa: A case report. *International Journal of STD and AIDS*. 30(5):519-21.

3. Peters, R.P.H, De Vos, L., **Maduna, L.D**, Mudau, M., Klausner, J. D., Kock, M. M. and Medina-Marino, A. 2017. Laboratory validation of Xpert *Chlamydia trachomatis*/*Neisseria gonorrhoeae* and *Trichomonas vaginalis* testing as performed by nurses at three primary health care facilities in South Africa. *Journal of Clinical Microbiology,* 55**,** 3563-5.

Published Abstracts

1. **Maduna L.D,** Kock M, Radebe O, Struthers, H., McIntyre, J., Peters, R.P.H. 2019. P647 Effective monotherapy due to high rate of azithromycin resistance in *Neisseria gonorrhoeae* infection in men in South Africa. *Sexually Transmitted Infections*, 95, A285-A285

2. Laumen, J., Alphen, L. V., **Maduna, L.D**, Medina-Marino, A., Mcintyre, J., Klausner, J., Kock, M. and Peters, R.P.H. 2019. P617 Molecular typing of *Mycoplasma genitalium* shows a diverse epidemic with limited azithromycin resistance in South Africa. *Sexually Transmitted Infections,* 95**,** A273-A273.

Manuscripts in preparation

1. **Maduna, L.D**., Kock, M.M., Brian M.J.W. van der Veer., Radebe, O., McIntrye, J., Lieke B van Alphen., Peters, R.P.H. 2019. Genomic analysis of *Neisseria gonorrhoeae* infection among high risk men demonstrates high rates of resistance and a distinct epidemic in South Africa

2. **Maduna, L.D**., Peters, R.P.H., Mogale, G., Dube, R., Kingsburgh, C., Strydom, KA., Coetzee, J., Kock M.M. 2019. High rates of antimicrobial resistance in *Neisseria gonorrhoeae* and *Mycoplasma genitalium* infections in the private healthcare sector in South Africa
3. Peters, R.P.H., **Maduna, L.D.,** Mudau, M., McIntrye, J., Klausner, J.D., Kock M.M., Medina-Marino, A. 2019. Treatment response of genital LGV biovar *Chlamydia trachomatis* infection to single-dose azithromycin in South African women

Conference presentations

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1. University of Pretoria-Faculty day 2019: 1st prize winner for best oral presentation
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Summary

Antimicrobial resistance (AMR) in *Neisseria gonorrhoeae* has emerged worldwide and treatment failures of ceftriaxone and azithromycin; the last remaining empirical first-line therapy for gonorrhoea, are reported. However, there is little information about the situation in South Africa where syndromic management is used to treat sexually transmitted infections (STIs). The purpose of this PhD study was to investigate the occurrence of AMR and molecular epidemiology of *N. gonorrhoeae* infections in high-risk men from the public and private healthcare sectors in South Africa.

The study included specimens from two study groups of participants: (1) core transmission groups of men-who-have-sex-with-men and men with recurrent discharge accessing sexual health services in Johannesburg; (2) *N. gonorrhoeae* and *Mycoplasma genitalium* isolates from patients accessing the private healthcare services. Urine and urethral swabs were collected from men for *N. gonorrhoeae* culture followed by antimicrobial susceptibility testing (AST). Molecular diagnostics for curable STIs was performed, including *M. genitalium* as an important coinfection, and whole genome sequencing (WGS) of gonococcal isolates to identify genetic resistance mutations and describe gonococcal populations. Antimicrobial susceptibility testing was performed on *N. gonorrhoeae* isolates obtained from private sector followed by genotyping using the *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) method. Melting curve and sequence analysis were performed on *M. genitalium* strains for detection of macrolide

resistance-associated mutations in the 23S rRNA. The quinolone resistance-determining regions of the *parC* and *gyrA* genes were also sequenced.

Neisseria gonorrhoeae was the main aetiology (82%) of urethral discharge in male core transmission groups. High rates of AMR to tetracycline, ciprofloxacin and penicillin were detected among gonococcal isolates and azithromycin resistance was identified in 15% of the gonococcal isolates obtained from public but not in the private sector. There was no resistance to spectinomycin and cephalosporins found. Resistance to azithromycin was associated with an A39T alteration in *mtrR* and A deletions in the *mtrR* promoter. Amino acid alterations in GyrA (S91F, D95G, D95A) and ParC (D86N, S87N, E91G) were associated with ciprofloxacin resistance. Tetracycline resistant isolates harboured a *tetM* plasmid and had mutations in the *rpsJ* gene. Whole genome sequencing analysis of the gonococcal isolates revealed a wide diverse epidemic with a substantial number of novel *N. gonorrhoeae* sequence typing for antimicrobial resistance (NG-STAR) (70%) and NG-MAST (70%) sequence types (STs) identified. *Neisseria gonorrhoeae* strains from the private sector were genetically diverse and a substantial number of novel NG-MAST STs (83%) were identified. A high rate of azithromycin resistance was detected (19%) in the private sector but not in the public sector in *M. genitalium* strains harbouring mutations in the 23S rRNA. Fluoroquinolone resistance (2%) was detected in *M. genitalium* isolates harbouring mutations in the *gryA* and *parC* genes.

The results show AMR in *N. gonorrhoeae,* and in the important coinfection *M. genitalium*, has emerged in South Africa. This work highlights that WGS can be successfully implemented in a resource-constraint setting for microbiological characterisation of gonococcal populations and their mechanisms of resistance. There is a need to urgently introduce diagnostics for STI care and scale-up surveillance for early detection of emerging AMR in STIs, in both public and the private sector in South Africa.

Key words: *Neisseria gonorrhoeae*; Antimicrobial resistance; Sexually transmitted infections; Whole genome sequencing; Core transmission groups; Azithromycin resistance*; Mycoplasma genitalium*; Fluoroquinolone resistance; Macrolide resistance; public healthcare sector; private healthcare sector

CHAPTER 1

Introduction

Gonorrhoea is a major sexually transmitted infection (STI), with an estimated 87 million gonococcal infections worldwide annually (World Health organization, 2018). The Sub-Saharan Africa has highest incidence rates of gonorrhoea worldwide with approximately 50 cases per 1000 men and 41 cases per 1000 women (Rowley *et al* 2019; Unemo *et al* 2019a). The aetiological agent is *N. gonorrhoeae*, an aerobic Gram-negative diplococcus with fastidious growth requirements. *Neisseria gonorrhoeae* spreads through unprotected sexual intercourse and selectively infects the mucosal surfaces of the urethra, endocervix, rectum, pharynx and conjunctiva; often causing a mucopurulent inflammatory reaction at these sites (Hook III and Handsfield, 1999). Gonococcal infections are usually acute and symptomatic in men, with the disease manifesting as purulent or mucopurulent discharge often accompanied by painful urination and testicular pain (Barry and Klausner, 2009; Unemo and Shafer, 2014). Undetected or not treated urogenital or rectal infections lead to complications such as epididymitis, penile oedema, urethral stricture, proctitis and reduced fertility (Handsfield *et al.*, 1974; Tsevat *et al.*, 2017). Disseminated gonococcal infections can lead to septic arthritis, meningitis and endocarditis (Hook III and Handsfield, 1999). Importantly, gonococcal infection like many other STIs, substantially enhances human immunodeficiency virus (HIV) transmission and susceptibility (Grosskurth *et al.*, 1995; Fleming and Wasserheit, 1999; Tapsall *et al.*, 2009).

Antibiotic therapy is the mainstay for management of gonorrhoea, as there are currently no vaccines in the (short-term) pipeline. However, *N. gonorrhoeae* has the propensity to acquire and sustain antimicrobial resistance (AMR). *Neisseria gonorrhoeae* has sequentially developed resistance to multiple antimicrobials that were previously recommended as first-line monotherapy since the 1930s (Unemo and Nicholas, 2012; Lewis, 2014). In response to emerging antimicrobial resistance, the Centres for Disease Control and Prevention (CDC) had declared emergence of antimicrobial resistant *N. gonorrhoeae* one of the top three global AMR urgent threats to public health in 2012 (Kirkcaldy *et al.*, 2012). Recently, the WHO in 2017 published its first ever list of Antibiotic-Resistant Bacteria to Guide Research, Discovery and Development of New antibiotics and has ranked antimicrobial resistant *N. gonorrhoeae* a "Priority tier 2", microorganism (Tacconelli *et al.*, 2017).

Currently combination therapy of ceftriaxone plus azithromycin is the last remaining empirical first-line therapy for gonorrhoea in many countries. However, over the last decade new multi-drug resistant (MDR) and extensively drug resistant (XDR) *N. gonorrhoeae* strains have emerged globally (Buono *et al.*, 2014; Unemo *et al.*, 2019b). Treatment failures and gonococcal strains with reduced susceptibility to extended spectrum cephalosporins have been reported across Asia, Europe, North America and South Africa (Lewis *et al.*, 2013; Wi *et al.*, 2017; Unemo *et al.*, 2019b). Of major concern is that, the first *N. gonorrhoeae* superbug strains belonging to the FC428 clone resistant to ceftriaxone plus high-level azithromycin resistance were reported in 2018 (Eyre *et al.*, 2018; Whiley *et al.*, 2018). These reports highlight an alarming waning efficacy of the current dual therapy ceftriaxone plus azithromycin, the last remaining treatment option for gonorrhoea.

Consequently, the WHO in 2012 issued a global action plan to control the spread and impact of AMR *N. gonorrhoeae* (WHO, 2012; Wi *et al.*, 2017). Some of the key priorities in the WHO global action plans are advocacy for increased awareness on correct use of antibiotics especially in key populations such as men who have sex with men (MSM) and commercial sex workers (CSW) and strengthened quality assured AMR surveillance particularly in countries with a high burden of *N. gonorrhoeae* infections (WHO, 2012). In South Africa, the syndromic management approach is used for treatment of sexually transmitted infections (STIs) in the public healthcare sector (accessed by 85%-90% of population) (Department of Health, 2015; Statistics South Africa, 2018). However, in the private sector (accessed by 10%-15% of population) there is access to laboratory diagnostics to guide treatment and the syndromic management is used as well. The syndromic management approach has well documented limitations such as the inability to detect asymptomatic infections, lack of antimicrobial susceptibility testing, unnecessary and inappropriate use of antibiotics (overtreatment) with the risk of antimicrobial resistance development, and lack of laboratory infrastructure and isolates for surveillance (White *et al.*, 2008; Garrett *et al.*, 2018).

Gonococcal AMR surveillance and the molecular characterisation of the mechanisms underlying these resistance phenotypes are essential in order to establish correct empirical therapies, as well as to describe the emergence of new mechanisms in local bacterial populations (Whiley *et al.*, 2012). Whole genome sequencing (WGS) provides robust evidence of the current AMR burden and essential surveillance data where there is none. An added benefit of WGS is that genomic data is rapidly becoming the gold standard for transmission

profiling and this would aid identification of sexual networks and provides a global context for studying the evolution of *N. gonorrhoeae* and its transmission routes (Mortimer and Grad, 2018). The best approach to obtain insight in the antimicrobial resistant *N. gonorrhoeae* epidemic in South Africa is to investigate core transmission groups such as MSM and men with multiple gonorrhoea episodes. Many studies have shown that antimicrobial resistant *N. gonorrhoeae* is strongly associated with MSM (Cole *et al.*, 2012; Lewis, 2013; Kenyon and Osbak, 2014; Xiridou *et al.*, 2014). In addition, men with repeat gonorrhoea episodes are at higher risk of acquiring antimicrobial resistant *N. gonorrhoeae* infection and these individuals may provide a sustained reservoir of antimicrobial resistant *N. gonorrhoeae* transmission within a community and their sexual networks (Fung *et al.*, 2007; Bautista *et al.*, 2016).

Individuals with *N. gonorrhoeae* infections are at risk for other STIs (Tapsall and Kinchington, 1996). The CDC recommends individuals diagnosed with gonococcal infection should be tested for other STIs such as *Chlamydia trachomatis*, *Trichomonas vaginalis* and *Mycoplasma genitalium* (Workowski and Bolan, 2015). *M. genitalium* is associated with urogenital tract infections in men and women, with a symptomatology that resembles *C. trachomatis* infection (Jensen, 2017). Several studies have shown a high number of concurrent *N. gonorrhoeae* and *M. genitalium* infections (Getman *et al.*, 2016; Fernández-Huerta and Espasa, 2019). A recent study among MSM in Australia found that 17% had a dual infection with *M. genitalium* and *N. gonorrhoeae* (Read *et al.*, 2019) The emergence of antimicrobial resistance in *M. genitalium* is linked to the widespread use of azithromycin for its treatment (Bissessor *et al.*, 2015).

The emergence of gonococcal strains resistant to ceftriaxone and azithromycin presents a substantial public health threat globally, and the prospect of untreatable gonorrhoea in the near future is a real possibility as there are currently no vaccines in the pipeline or new antibiotics licensed to manage *N. gonorrhoeae* infection of untreatable gonorrhoea (Unemo and Shafer, 2014). The reasons for the emergence of AMR *N. gonorrhoeae* are multifactorial and are all present in South Africa: (i) inadequate antimicrobial stewardship leading to selection pressure imposed by widespread use of broad-spectrum antimicrobials, (ii) high-risk sexual behaviour increases chances of *N. gonorrhoeae* infection and transmission, and (iii) inadequate partner notification is associated with high rates of repeat diagnoses with STI. Moreover, as is the case in other African countries, there is limited data on antimicrobial resistant *N. gonorrhoeae* infections in South Africa to fully understand the burden of antimicrobial resistant gonorrhoea.

Historically core groups have contributed to the emergence and spread of antimicrobial resistance in *N. gonorrhoeae* (Lewis, 2013) and such resistance, if present, would most likely emerge first in these core groups such as MSM and men with recurrent STIs in South Africa. Therefore, the study investigated the burden of antimicrobial resistant gonococcus among high risk men in South Africa.

Aim

The aim of this to study was to determine the occurrence, molecular epidemiology and antimicrobial resistance patterns of *Neisseria gonorrhoeae* infections among high risk men in Gauteng, South Africa.

Objectives

- To perform a multivariate analysis of demographic and behavioural factors as well as coinfections as associated with *N. gonorrhoeae* infection in high risk men.

- To determine phenotypic and genotypic antimicrobial resistance patterns of *N. gonorrhoeae* in relation to treatment regimen.

- To determine the distribution of *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) sequence types in relation to resistance and bio-behavioural data.

- To investigate molecular mechanisms of antibiotic resistance in *N. gonorrhoeae* isolates using whole genome sequencing.

- To detect macrolide resistance-associated mutations in the 23S rRNA gene by melt curve analysis and characterise macrolide resistance in *M. genitalium.*

- To assess antimicrobial resistance of *N. gonorrhoeae* and *M. genitalium* in the private health sector.

- To investigate the impact of storage temperature and transport time on the detection of *C. trachomatis, N. gonorrhoeae* and *T. vaginalis* infections using the GeneXpert® platform.

Outline of thesis

In **chapter 1** background information about the challenges faced in South Africa with understanding the existence and potential emergence of antimicrobial resistant *N. gonorrhoeae* infections was provided. **Chapter 2** contextualises the study through up-to-date review of the relevant literature. **Chapter 3** provides detailed analysis of the aetiology of STIs, phenotypic and genotypic antimicrobial resistance and molecular epidemiology of *N. gonorrhoeae* infections in high risk men. In **Chapter 4** a case of antimicrobial resistant *M. genitalium* infection, which could undermine the syndromic management in the treatment of STIs in South Africa was described. In **Chapter 5** data on a joint project with Maastricht University Medical Centre, the Netherlands on the molecular epidemiology of *M. genitalium* in South Africa is presented. **Chapter 6** provides information on antimicrobial resistance in *N. gonorrhoeae* and *M. genitalium* in the private healthcare sector in South Africa. **Chapter 7** provides unique data on the logistics of implementation of laboratory diagnostics of STIs in resource constraint settings using molecular tests. This information is very useful to policy makers in implementation of the GeneXpert® (Cepheid, USA) platform for testing STIs in resource-constraints settings. Finally, in **Chapter 8** an overview of the main findings of this thesis are presented and the implications on the public health.

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CHAPTER 2

Literature review

This literature review contextualises the present PhD study in the relevant literature. The focus of this literature review is on the epidemiology of *Neisseria gonorrhoeae* infections and their clinical manifestations, on the history of *N. gonorrhoeae* antimicrobial regimens and development of resistance to them, on the genetic resistance mutations of *N. gonorrhoeae* to previously and currently recommended antimicrobials; on the detection and genotyping of resistant gonococcal strains and on future treatment regimens for gonorrhoea.

2.1 *Neisseria gonorrhoeae* epidemiology

Neisseria gonorrhoeae is an aerobic fastidious Gram-negative, oxidase positive, intracellular diplococcus (Unemo and Shafer, 2014). *Neisseria gonorrhoeae* is the aetiologic agent for the sexually transmitted infection (STI) gonorrhoea (Barry and Klausner, 2009; Unemo and Shafer, 2014). Gonorrhoea is a major public health concern, the World Health Organization (WHO) estimated 86.9 million infections among 15 to 49-year olds in 2016 (WHO, 2018). Sub-Saharan Africa has the highest incidence rates of gonorrhoea worldwide, i.e. with an estimated 41 new infections per 1000 women and 50 infections per 1000 men in 2016 (Rowley *et al.,* 2019; Unemo *et al* 2019). However, only a small number of gonorrhoea cases is confirmed in laboratories in Africa due to the absence of laboratory diagnostics and the use of syndromic management for treatment of STIs (Ndowa *et al.*, 2013; Unemo and Dillon, 2014; Alirol *et al.*, 2017).

In South Africa, the estimated prevalence of gonorrhoea was 6.6% (95% confidence interval [CI]: 3.8-10.8%) and 3.5% (1.7-6.1%) in women and men respectively in 2017 (Kularatne *et al.*, 2018b). These WHO spectrum model estimates translated into 2.3 (1.1-5.0) million and 2.2 (1.1-3.8) incident gonorrhoea cases in women and men respectively (Kularatne *et al.*, 2018b). Gonorrhoea is the most common aetiology of male urethritis syndrome (MUS) in South Africa accounting for 70 to 85% of MUS cases (Mhlongo *et al.*, 2010; Kularatne *et al.*, 2018a). In addition, there is a high burden of gonorrhoea in men who have sex with men (MSM) in South Africa and elsewhere in Africa compared to the heterosexuals (Muraguri *et al.*, 2012; Rebe *et al.*, 2015; Van Liere *et al.*, 2019).

A mathematical modelling has shown sustained propagation of gonorrhoea within a population is strongly linked with high-risk individuals who are frequently and repeatedly infected with *N. gonorrhoeae;* these individuals known as core transmission groups are characterised by high numbers of sexual partner change, concurrent partners and repeat infections (WHO, 2007; Chan *et al.*, 2012; Hethcote and Yorke, 2014). As the gonorrhoea epidemic is sustained, *N. gonorrhoeae* infection is spread into lower-risk populations (bridging populations), thereby presenting an important sexual link between the core transmission groups and the general population (Figure 2.1) (WHO, 2007). Historically antimicrobial resistant *N. gonorrhoeae* has emerged first in core transmission groups such as MSM and CSWs (Lewis, 2013). Furthermore, studies have shown MSM are significantly at risk of acquiring *N. gonorrhoeae* resistant to multiple classes of antimicrobials (Kirkcaldy *et al.*, 2013; Kenyon and Schwartz, 2018).

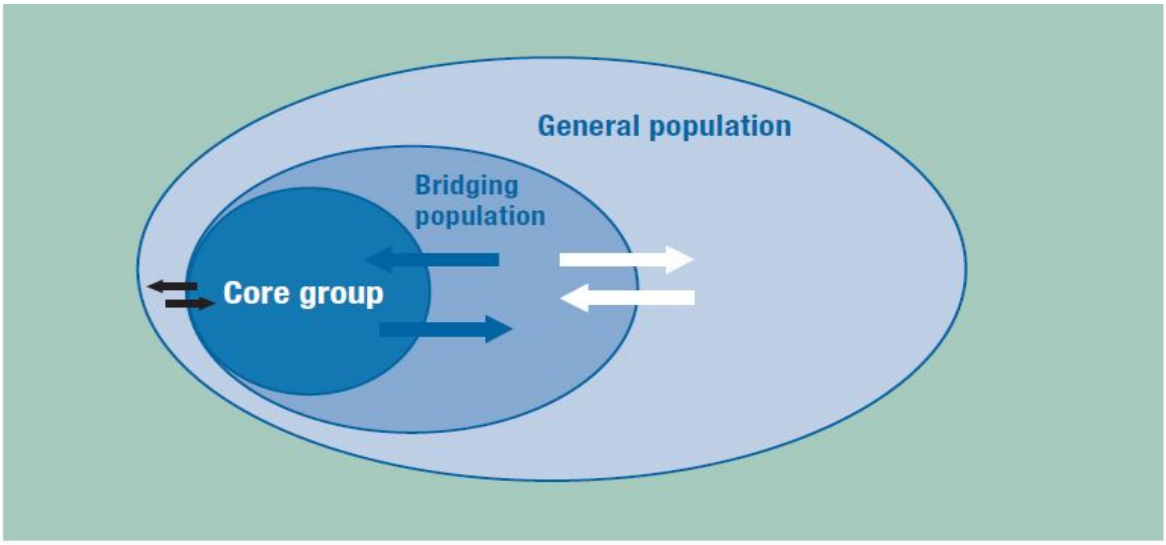

Figure 2.1 Transmission dynamics of sexually transmitted infections at the population level (Source: WHO, 2007)

2.2 Clinical manifestations

Neisseria gonorrhoeae is an obligate human pathogen; it selectively infects the epithelial cells of mucosal surfaces of the human urogenital tract, pharynx and rectum during unprotected sexual contact (Golden and Handsfield, 2012). In men symptomatic gonococcal urethral infections are typically characterised by purulent or mucopurulent discharge often accompanied by dysuria and sometimes testicular pain (Barry and Klausner, 2009). As many as 40% of urogenital infections in men will be asymptomatic which can lead to complications such as epididymitis, reduced fertility, and ultimately urethral stricture (Handsfield *et al.*, 1974; Alirol *et al.*, 2017; Tsevat *et al.*, 2017). Urogenital infections in women usually present as cervicitis,

however up to 50% of women will be asymptomatic (Unemo *et al.*, 2017). Complications in women include pelvic inflammatory disease, reproductive tract complications (infertility, risk of ectopic pregnancy) and adverse pregnancy outcomes (pre-term delivery, low birthweight neonates and stillbirth) (Liu *et al.*, 2013; Tsevat *et al.*, 2017). New-borns can acquire a gonococcal infection during transit of the birth canal presenting as ophthalmia neonatorum and that may lead to blindness if left untreated (Unemo and Shafer, 2014). Disseminated gonococcal infection in both sexes can lead to septic arthritis, meningitis and endocarditis (Unemo and Shafer, 2014).

Extra-genital infections can occur at any mucosal surface exposed during sexual contact including the rectum, pharynx and conjunctiva; often causing a mucopurulent inflammatory reaction (Koedijk *et al.*, 2012; Van Liere *et al.*, 2014; Peters *et al.*, 2016). Anorectal infections are frequently asymptomatic, manifest as rectal bleeding and pain or discharge (Kent *et al.*, 2005). Most pharyngeal infections are asymptomatic and predominant among MSM but can occur in both sexes depending on the sexual practice, usually manifesting as pharyngitis or a sore throat (Kent *et al.*, 2005; Unemo and Shafer, 2014). Since the commensal *Neisseria* species are more abundant in the oropharynx than in the urethral or cervical niches, it is thought that acquisition of resistance traits through horizontal gene transfer will likely occur in the oropharynx (Unemo and Shafer, 2014). Importantly, gonococcal infection substantially enhances the risk of acquisition and transmission of human immunodeficiency virus (HIV) up to five-fold (Cohen *et al.*, 1997; Fleming and Wasserheit, 1999; Barry and Klausner, 2009; Tapsall *et al.*, 2009).

2.3 *Neisseria gonorrhoeae* infection and other Sexually Transmitted Infections

Individuals with gonococcal infections are frequently infected with other STI pathogens such as *C. trachomatis*, *M. genitalium* and *T. vaginalis* (Tapsall and Kinchington, 1996). *Chlamydia trachomatis* is a common STI globally, associated with genital and urogenital tract infection in women and men (Vos *et al.*, 2016). Lymphogranuloma venereum, an invasive variant of *C. trachomatis* is the leading cause of proctitis among MSM worldwide (De Vrieze and De Vries, 2014). The majority of chlamydial infections in women are asymptomatic and may lead to reproductive tract complications (Haggerty *et al.*, 2010). Studies have documented high co-infections rates with *N. gonorrhoeae* and *C. trachomatis* infections (Dicker *et al.*, 2003; Miller *et al.*, 2004; Donati *et al.*, 2009). On this basis, empiric therapy for *C. trachomatis* is recommended upon detection of *N. gonorrhoeae* or if suspected (Gilbert *et al.*, 2003; Vonck *et*

al., 2011). *Trichomonas vaginalis,* a protozoan, is the most common curable STI worldwide, causes vaginal discharge in females and urethritis in men (Johnston and Mabey, 2008). There is a high burden of *T. vaginalis* infection among women from disadvantaged populations in both well-resourced and resource-constraint settings (Hirt and Sherrard, 2015).

2.3.1 *Mycoplasma genitalium* infections and antimicrobial resistance

Mycoplasma genitalium is a fastidious small bacterium, an important cause of non-gonococcal and non-chlamydial urethritis in men and cervicitis in women (Daley *et al.,* 2014; Lis *et al.,* 2015). The disease presentation and symptoms are indistinguishable from those of *C. trachomatis* (Jensen, 2017). Untreated *M. genitalium* cervicitis is associated with pelvic inflammatory disease, pregnancy complications (preterm birth, spontaneous abortion) and infertility in women (Taylor-Robinson and Jensen, 2011; Daley *et al.*, 2014). In addition, *M. genitalium* poses a significant public health as individuals infected with the bacterium are at risk for both acquiring and transmitting HIV (Dietz *et al*., 2018). In symptomatic men with urethritis, *M. genitalium* infections account for approximately 1% to 3% cases (Taylor-Robinson and Jensen, 2011; Baumann *et al.*, 2018). The prevalence of *M. genitalium* infection in South Africa was reported as 8.1% in men and 8.6% in women in the sentinel aetiological surveillance (Mhlongo *et al.*, 2010; Kufa *et al.*, 2018). *M. genitalium* lacks peptidoglycan and thus results in insensitivity to antibiotics which target cell wall synthesis such as β-lactam antibiotics (Tien *et al*., 2019). The recommended first line empiric therapy for *M. genitalium* infections is a macrolide, azithromycin in many settings (Couldwell and Lewis, 2015; Unemo and Jensen, 2017). Moxifloxacin, a fluoroquinolone is a recommended second-line empiric agent for treatment of *M. genitalium* infections (Unemo and Jensen, 2017). However increasing rates of antimicrobial resistance to these agents has compromised treatment of *M. genitalium* infections worldwide and treatment failures have been reported (Bradshaw *et al.*, 2008; Bissessor *et al.*, 2014; Couldwell and Lewis, 2015). Though antimicrobial resistance has emerged in other STI pathogens like *N. gonorrhoeae,* antibiotic resistance in *M. genitalium* has rapidly emerged in a speed belying its small size (Couldwell and Lewis, 2015; Sethi *et al*., 2017).

Macrolide resistance in *M. genitalium* infection, an important coinfection associated with *N. gonorrhoeae* infection, is emerging worldwide (Couldwell and Lewis, 2015). Macrolide resistance in *M. genitalium* was first reported in Australia in 2008 and since that time, the efficacy of macrolides in treatment of *M. genitalium* infections has diminished; with current the

resistance rates estimated at 30-100% worldwide (Tien *et al.*, 2019). Development of azithromycin resistance in *M. genitalium* readily occurs via antibiotic selection pressure resulting in the selection of mutants or the acquisition of single base mutations (Tien *et al.*, 2019). The waning efficacy of azithromycin and treatment failures in *M. genitalium* infections is strongly associated with mutations in region V of the 23S rRNA gene, commonly nucleotide substitutions at A2071 and A2072 (*M. genitalium* numbering) (Jensen *et al.*, 2008; Couldwell and Lewis, 2015). Furthermore, macrolide resistance associated mutations in the L4 and L22 which alter ribosomal proteins have been described in *M. genitalium* isolates with low level resistance (Jensen *et al.,* 2008).

Resistance to fluoroquinolones in *M. genitalium* infections is mediated by single nucleotide polymorphisms in the quinolone resistance determining region of topoisomerase IV *par*C gene and DNA gyrase (*gryA* gene), frequently at amino acid positions Ser83 and Asp87 (*M. genitalium* numbering) (Deguchi *et al.*, 2017; Hamasuna *et al.*, 2018). Cultured *M. genitalium* strains with high minimum inhibitory concentration values have been found harbour single nucleotide positions (SNPS) resulting in amino acid change at Ser83→Ile in ParC and Asp99→Asn, Gly93→Cys and Met95→Ile in GyrA (Van Der Schalk *et al.*, 2020).

Molecular methods have been increasing used for detection of *M. genitalium* in clinical specimens due to its fastidious requirements for culture *in vitro* (Getman *et al*., 2016*)*. The use of nucleic acid amplification tests for detection (NAATS) of *M. genitalium* genomic DNA and transcription-mediated amplification for detection of *M. genitalium* 16S rRNA has transformed our understanding of the epidemiology and antimicrobial resistance of *M. genitalium* infections (Getman *et al.*, 2016). Furthermore, the use of NAATS in detection of resistance mutations in DNA extracts have aided the correlation of genotypic resistance and clinical outcomes (Sethi et al., 2017).

2.4 History of *Neisseria gonorrhoeae* antimicrobial treatment and development of resistance

There are no vaccines currently available against *N. gonorrhoeae*, antibiotics are the cornerstone for gonorrhoea therapy (Lewis, 2019). However, *N. gonorrhoeae* has developed antibiotic resistance at alarming rates to multiple antimicrobials which have been used for gonorrhoea therapy (penicillins, sulphonamides and trimethoprim combinations, tetracyclines,

spectinomycin, fluoroquinolones, macrolides and cephalosporins) (Figure 2.2) and now the threat of untreatable gonorrhoea has become real (Unemo and Shafer, 2014).

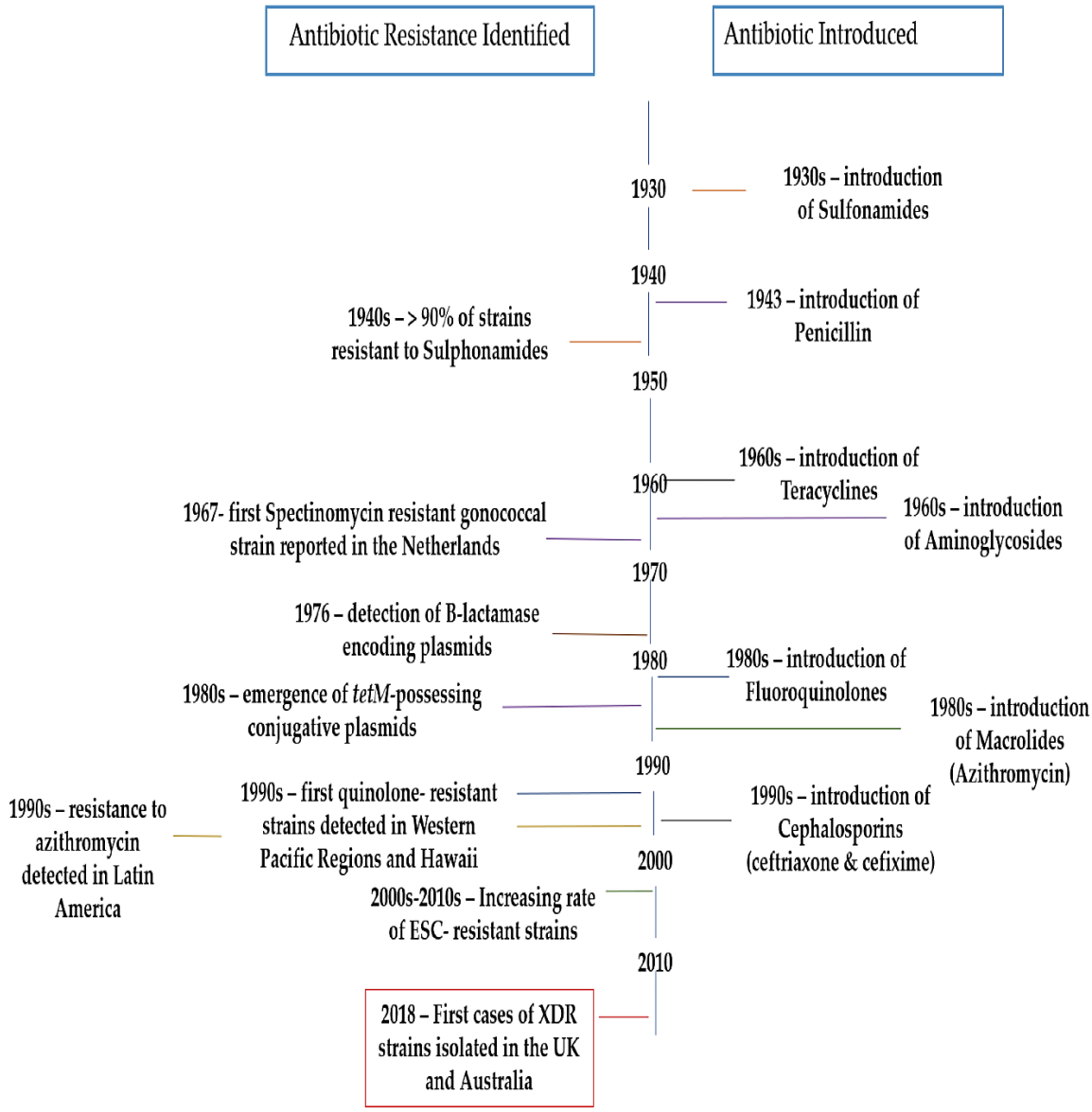

Figure 2.2 History of discovery of antimicrobials and evolution of resistance in *Neisseria gonorrhoeae*, including the emergence of genetic resistance determinants, internationally (Source: Suay-García and Pérez-Gracia, 2018).

2.4.1 Sulphonamides

Prontosil, one of the first sulphonamides to be used in gonorrhoea therapy was discovered in 1935 by Gerhard Domagk (Dees and Colston, 1937; Kampmeier, 1983). Sulphonamides inhibit folic acid biosynthesis that is essential in maintaining the gonococcus cell wall by acting as a competitive substrate of the enzyme dihydropteroate synthetase (Johnson and Morse, 1988;

Costa-Lourenco *et al.*, 2017). Initially, sulphonamides achieved a cure rate between 80% to 90% for gonococcal infections (Lewis, 2010; Unemo and Shafer, 2014). Over time, clinical resistance of gonococci to sulphonamides emerged and by the end of the 1940s more than 90% of *N. gonorrhoeae* were resistant to sulphonamides (Lewis, 2010; Unemo and Shafer, 2014). Subsequently, a combination therapy of trimethoprim and sulfamethoxazole (e.g Bactrim) was introduced to improve the efficacy of sulphonamides in the treatment of gonococcal infections and was used for many decades until resistance developed (Austin *et al.*, 1973; Lawrence *et al.*, 1973).

2.4.2 Penicillins

Cecil Paine in 1930 used an extract of the fungus *Penicillum notatum,* for the treatment of gonococcal ophthalmia neonatorum (Wainwright and Swan, 1986). However, penicillin was only introduced as therapy for gonorrhoea a decade later in 1943 and was primarily reserved for sulphonamide treatment failures (Van Slyke *et al.*, 1943; Unemo and Shafer, 2014). Thereafter, penicillin became the mainstay of treatment for gonococcal urethritis with an efficacy of more than 95%, thereby ultimately replacing sulphonamides as first-line therapy of gonorrhoea (Van Slyke *et al.*, 1943; Sternberg and Turner, 1944). Over time, gonococci became less susceptible to penicillin due to cumulative chromosomal mutations and therefore higher doses of penicillin were required to achieve cure (Jaffe *et al.*, 1976; Unemo and Shafer, 2014). The number of gonococcal strains exhibiting resistance to penicillin progressively increased (Jaffe *et al.*, 1976; Shafer *et al.*, 2010); with the effective penicillin dose for gonococcal infections increasing up to 24-fold, from the initial 200,000 units in the 1950s to 4.8 million units in the 1970s (Martin *et al.*, 1970; Costa-Lourenco *et al.*, 2017). The emergence of penicillinase producing *N. gonorrhoeae* in Africa, Asia, North America and Europe in 1976 led to the demise of penicillin as a antibiotic of choice for treatment of gonorrhoea (Lewis, 2010). Penicillinase producing *N. gonorrhoeae* harboured plasmids encoding a Temoniera (TEM)-1-type β-lactamase (Phillips, 1976). The TEM-1-type β-lactamase plasmids were readily transferable between *N. gonorrhoeae* strains and penicillinase producing *N. gonorrhoeae* and therefore spreading rapidly (Phillips, 1976; Dillon and Yeung, 1989).

2.4.3 Tetracycline

Tetracyclines were discovered in the late 1940s and were originally used to treat gonorrhoea in patients allergic to penicillin (Reyn *et al.*, 1958). However, over time, gonococci became less

susceptible to tetracyclines and chromosomal resistance soon emerged. Tetracycline-resistant *N. gonorrhoeae* due to plasmid-mediated expression of *tetM* gene emerged in the 1980s and spread globally (Morse *et al.*, 1986). Soon after, tetracycline was discontinued as a first-line treatment of gonococcus in many countries worldwide (Lewis, 2010).

2.4.4 Spectinomycin and gentamicin

Spectinomycin, is an aminocyclitol produced naturally by many microorganisms (e.g cyanobacteria) and was developed in the early 1960s for treatment of gonorrhoea (Easmon *et al.*, 1985). It became useful in the treatment of the gonococcus after the emergence of penicillinase producing *N. gonorrhoeae* although it is less efficacious against pharyngeal gonorrhoea (Judson *et al.*, 1985). Shortly after its introduction into clinical use, spectinomycin resistant gonorrhoeae emerged. In 1967, spectinomycin resistance was reported in a penicillin susceptible gonococcus strain in the Netherlands (Stolz *et al.*, 1975) and later in a Penicillinase producing *N. gonorrhoeae* strain in the Philippines in 1981 (Ashford *et al.*, 1981).

Spectinomycin was introduced as antibiotic of choice for treatment of gonococcus in the United States (US) military personnel in South Korea in 1981 (Berg and Harrison, 1981; Boslego *et al.*, 1987). However, within 4 years of its introduction, an 8.2% clinical failure rate was reported and was soon abandoned as first line treatment for gonorrhoea globally (Boslego *et al.*, 1987). Currently gonococcal resistance to spectinomycin is rare worldwide (Unemo *et al.*, 2019a). Gentamicin has successfully been used in the treatment of gonorrhoea since 1993 in Malawi as part of the syndromic management approach (Kamanga *et al.*, 2010). Even though, clinical data on resistance is generally lacking in resource constraints settings; studies in Malawi have shown the gonococci remains 100% susceptible to gentamicin after two decades of first-line use (Daly *et al.*, 1997; Brown *et al.*, 2010).

2.4.5 Fluoroquinolones

In the late 1980s fluoroquinolones, such as ciprofloxacin and ofloxacin were introduced in the treatment against *N. gonorrhoeae* and became very popular alternatives to penicillin (CDC, 1987; Newman *et al.*, 2004). Due to excellent safety and efficacy at all anatomical sites including the oropharynx, ciprofloxacin was recommended as the first line therapy for treatment of gonorrhoea globally, though not suitable for pregnant women or children (Lewis, 2010; Patel *et al.*, 2011). Initially, low doses of ciprofloxacin (250 mg) were used in the treatment of

gonorrhoea but by the 1990s many therapeutic failures were already reported (Gransden *et al.*, 1990). Consequently, the recommended ciprofloxacin treatment dose was increased to 500 mg; however, over time resistance quickly developed, firstly in the Asian-Pacific region (Tanaka *et al.*, 2000; Unemo and Shafer, 2011). Subsequently, fluoroquinolones were abandoned as the first-line treatment of gonorrhoea in most Asian-Western Pacific countries in the mid to late 1990s (Tanaka *et al.*, 1994; Ray *et al.*, 2005). Fluoroquinolone resistant strains soon spread internationally and in the United Kingdom (UK) and the US these strains were prevalent among MSM (CDC, 2004; Martin *et al.*, 2005). Subsequently, in 2007 the CDC withdrew ciprofloxacin as the recommended treatment for gonorrhoea (Dowell *et al.*, 2012). Ciprofloxacin was then abandoned as the first-line treatment in many countries in Asia, Europe and some parts of Africa (Figure 2.2) in the early to mid-2000s due to high levels of fluoroquinolone resistance (Unemo and Shafer, 2011; Suay-García and Pérez-Gracia, 2018). Currently, a high proportion of gonococcal strains still exhibit clinical resistance to fluoroquinolones worldwide (Martin *et al.*, 2006; Bala *et al.*, 2013; Dillon *et al.*, 2013; Ndowa *et al.*, 2013; Unemo *et al* 2019a).

2.4.6 Macrolides

Erythromycin, the first antibiotic of the macrolide class was isolated from the soil microorganism *Saccharopolyspora erythraea* in 1952 (Lewis, 2010). Clinical and susceptibility data demonstrated that erythromycin performs poorly against the treatment of gonorrhoea (Brown *et al.*, 1977). Azithromycin was manufactured in 1980, has substantially higher efficacy against *N. gonorrhoeae* as compared to erythromycin and can be given as single dose treatment (Lewis, 2010). Following the widespread use of azithromycin in the treatment of bacterial STIs in Latin America, decreased susceptibility and resistance of gonococcal strains was reported in the mid-to-late 1990s (Dillon *et al.*, 2013; Unemo and Shafer, 2014). Soon afterwards, there was an emergence of azithromycin resistance in many countries where it was recommended for treatment of gonorrhoea and *C. trachomatis* infections (Dillon *et al.*, 2013; Lahra *et al.*, 2013). *N. gonorrhoeae* strains exhibiting high level resistance to azithromycin (minimum inhibitory concentration (MICs) ≥ 256 mg/L) have been reported from Argentina, Iceland, United Kingdom, Czech Republic, United States, China, Ireland and Italy (Chisholm *et al.*, 2009; Galarza *et al.*, 2009; Katz *et al.*, 2011; Ni *et al.*, 2016; Day *et al.*, 2018). Furthermore, an outbreak of 70 cases of *N. gonorrhoeae* infections with high level azithromycin resistant *N. gonorrhoeae* was reported in the UK, between November 2014 and February 2015, where

azithromycin was used in combination with ceftriaxone for treatment of gonorrhoea (Fifer *et al.*, 2019). Thirty- seven of the azithromycin resistant isolates in that outbreak belonged to the same *N. gonorrhoeae* Multiantigen Sequence Typing (NG-MAST) sequence type (ST): number 9768 (Fifer *et al.*, 2018). Subsequently, due to the concerning high rates of high-level azithromycin resistance in the UK, gonorrhoea treatment guidelines were revised in 2019 in the UK; dual therapy was abandoned and a higher dose of ceftriaxone 1 g intramuscularly (IM) was recommended as a monotherapy (Fifer *et al.*, 2019).

2.4.7 Cephalosporins

Following the development of resistance of *N. gonorrhoeae* towards fluoroquinolones and their demise, third-generation extended spectrum cephalosporins; both ceftriaxone (injectable) and cefixime (oral) were introduced in many countries as first-line therapy for gonorrhoea (Unemo and Shafer, 2011). Cefixime is the only oral extended spectrum cephalosporins which has met the criterion on the lower bound of the 95% confidence interval (CI) for effective treatment of pharyngeal gonorrhoea of the WHO recommended cure rate of 95% or greater (Lewis, 2010). Other oral cephalosporins have also been used when cefixime was not available in other countries e.g. cefditoren and celdinir in Japan, cefuroxime in the UK and cefpodoxime in the United States (Unemo and Shafer, 2014). Following widespread use of cefixime gonococcal strains with decreased susceptibility firstly emerged from Japan and then spread globally during the last two decades (Ito *et al.*, 2004). Additionally, these gonococcal strains were also resistant to other previously recommended first-line antibiotics (fluoroquinolones, tetracycline, macrolides and penicillin) resulting in treatment failure (Patel *et al.*, 2011).

At present, non-susceptible gonococcal strains with increased extended spectrum cephalosporins MICs are identified at an alarming rate in many countries in the world (Unemo; 2019a). Concerning is that cefixime treatment failures have been verified in Japan, Europe, Canada, South America (Unemo and Shafer, 2014) and South Africa (Lewis *et al.*, 2013). Additionally, these gonococcal strains are MDR with high-level resistance to fluoroquinolones, tetracyclines, macrolides and penicillins (Unemo and Shafer, 2014). Most concerning, XDR *N. gonorrhoeae* strains with high level resistance to all extended spectrum cephalosporins and additional resistance to fluoroquinolones as well as older drugs such as penicillins and tetracyclines have emerged among some of the high-risk populations, that is, CSW and MSM (Lewis, 2010; Unemo and Shafer, 2014).

A decade ago, the first XDR gonococcal 'superbug' H041 strain showing high-level resistance to cefixime (MIC= 8 µg/mL) and ceftriaxone (MIC= 2 to 4 µg/mL) was isolated from the oropharynx of a Japanese female CSW in Kyoto 2009 (Ohnishi, 2011). The H041 strain had the mosaic penicillin binding protein 2 (PBP-2); the target of extended spectrum cephalosporins and was resistant to all previously recommended antimicrobials. The H041 strain was assigned to multilocus sequence typing (MLST) ST 7363 and the NG-MAST ST 4220 (Ohnishi *et al.*, 2011). Fortunately, after intensive surveillance to determine the spread of the H041 strain, no additional infections were reported from this superbug. It has since been demonstrated that acquisition of mosaic PBP2 in MDR or XDR gonococcal strains leads to decreased biological fitness while unexpectedly developing other mutations to compensate the fitness (Vincent *et al.*, 2018; Unemo *et al.*, 2019a).

Two years later (2011) subsequent to the identification of the H041 strains, additional gonococcal XDR strains with increased ceftriaxone MICs were identified from the urethra of a MSM in France (strain F89, ceftriaxone MIC= 2 µg/mL) (Unemo *et al.*, 2012) and from the urethral and rectal samples of two MSM in a same sexual network (ceftriaxone MIC 1.5 µg/mL) in Spain (Ca´Mara *et al.*, 2012). All three European isolates are genetically related and shared the same NG-MAST ST 1407 but were unrelated to the Japanese H041 strain (NG-MAST ST 4220) (Unemo *et al.*, 2012). Furthermore, three European gonococcal isolates also belong to the superclone MLST ST 1901 which is circulating worldwide and is responsible for decreased susceptibility or resistance to extended spectrum cephalosporins (Unemo and Nicholas, 2012). Other *N. gonorrhoeae* strains with nonmosaic PBP2 can cause ceftriaxone resistance and have been identified in Asian countries (China, Korea and Vietnam) as well as in Argentina (Unemo *et al.*, 2019a).

The first failure of dual antimicrobial therapy (ceftriaxone and azithromycin) in treatment of pharyngeal gonorrhoea was reported in the UK in 2016; this was a male patient who contracted ceftriaxone and azithromycin resistant gonorrhoea in South-East Asia (Table 2.1) (Fifer *et al.*, 2016). Alarmingly, between February and April 2018, three other cases "super-resistant" gonococcal strains with combined ceftriaxone resistance (MIC=0.5 mg/L) and high-level azithromycin resistance (MIC>256 mg/L) were detected in UK (one case) (Eyre *et al.*, 2018) and Australia (two cases) (Whiley *et al.*, 2018). Two of these gonorrhoea cases were travel-associated and were contracted through recent travel to Southeast Asia. Whole genome sequencing analysis revealed that the strains were genetically related; they shared the same NG-

MAST ST 164848, MLST ST 12039 and *N. gonorrhoeae* Sequence Typing for Antimicrobial Resistance (NG-STAR) ST 996 (Jennison *et al.*, 2019). Furthermore, the strains also had a mosaic PBP2 conferring resistance to ceftriaxone (*penA* 60.001 allele) and they belonged to a clade of high-level azithromycin resistant strains (FC428 clone) which have A2059G mutations in all four alleles of the 23S rRNA gene known to be associated with high level azithromycin resistance (Jennison *et al.*, 2019; Unemo *et al.*, 2019a; Unemo *et al.*, 2019b). Recently, two cases of MDR *N. gonorrhoeae* were identified in France in 2019 (infection acquired in Cambodia) belonging to the successful ceftriaxone resistant FC428 Asian clone (Poncin *et al.*, 2019). Therefore, the emergence of gonococcal "superbug" strains resistant to ceftriaxone and azithromycin pose a substantial public health threat globally and we are facing very the real possibility of untreatable gonorrhoea.

Table 2.1 Main characteristics of *Neisseria gonorrhoeae* treatment failures of cefixime, ceftriaxone plus azithromycin or doxycycline

Country (no. cases)	Country of infection	Year	Therapy	MIC of CRO or AZM (mg/ L)	MLST	NG-MAST	NG-STAR	PBP2 allele	Site of Failure	References
Australia (n = 2	Australia	2007	CRO 250 mg x 1	0.016-0.03/ND	ND	2740	ND	ND	Pharynx	(Unemo, 2015)
Japan (n = 1)	Japan	2009	CRO 1 g x 1	4.0/1	7363	42220	226	37.001 (mosaic)	Pharynx	(Unemo, 2015)
Sweden (*n* = 1)	Japan	2010	CRO 250 mg x 1 & CRO 500 mg x 1	0.125–0.25/0.5	1901	1407	90	34.001 (mosaic)	Pharynx	(Unemo, 2015)
Australia (*n* = 1)	Australia	2010	CRO 500 mg x 1	0.030.06/0.25–0.5	ND	1407	ND	ND	Pharynx	(Unemo, 2015)
Slovenia (*n* = 1)	Serbia	2011	CRO 250 mg x 1	0.125/0.5	1901	1407	90	34.001 (mosaic)	Pharynx	(Unemo, 2015)
South Africa (n=2)	South Africa	2013	CXF 400 mg	0.125-0.064/1	1901	4822	ND	ND	Urogenital	(Lewis *et al.*, 2013)
UK (n=1)	Japan	2014	CRO 500 mg x 1 + AZM x 1 g	0.25/1	1901	12133	22	10.001 (mosaic)	Pharynx	(Unemo *et al.*, 2019b)
France (n=1)	France	2017	CRO 250 mg x 1 + DOX 100 mg x 2 daily, 7 days	0.5/0.5	1903	3435	233	60.001 (mosaic)	Pharynx	(Unemo *et al.*, 2019b)
UK (n=1)	Thailand	2018	CRO 1 g + DOX 100 mg x 2 daily, 7 days	0.5/>256	12039	16848	996	60.001 (mosaic)	Pharynx	(Eyre *et al.*, 2018)
UK (n=1)	UK	2018	CRO 1 g x 1 + DOX 100 mg x 2 daily, 7 days	1/0.5	1903	1614	233	60.001 (mosaic)	Rectum, urogenital tract	(Eyre *et al.*, 2018)
France (n=2)	Cambodia	2019	CRO 1 g x 1	0.5/0.5	13871	1086	233	60.001 (mosaic)	Urogenital	(Poncin *et al.*, 2019)

***Abbreviations:** AZM, azithromycin; CRO, ceftriaxone; DOX, doxycycline; CXF, cefixime; MIC, minimum inhibitory concentration; MLST, multilocus sequence typing; ND, not determined; NG-MAST, *N. gonorrhoeae* multi-antigen sequence typing; NG-STAR, *N. gonorrhoeae* sequence typing antimicrobial resistance; PBP2, penicillin-binding protein 2; ST, sequence type

2.5 Mechanisms of antimicrobial resistance in *Neisseria gonorrhoeae*

Neisseria gonorrhoeae is characterised by the propensity to acquire resistance to sequential antimicrobials recommended for treatment within 10 to 20 years of their introduction (Unemo and Shafer, 2014). *Neisseria gonorrhoeae* is naturally competent to acquire antimicrobial resistance by uptake and recombination of foreign DNA through transformation and hence, transfer of chromosomally encoded resistance genes occurs rapidly (Lewis, 2014). *N. gonorrhoeae* has utilised all known mechanisms of antimicrobial resistance such as: (i) the inactivation of the antibiotic, (ii) mutation of the drug binding sites, (iii) reduction of antibiotic influx, and (iii) increased drug efflux (Figure 2.3) (Barry and Klausner, 2009; Shaskolskiy *et al.*, 2016).

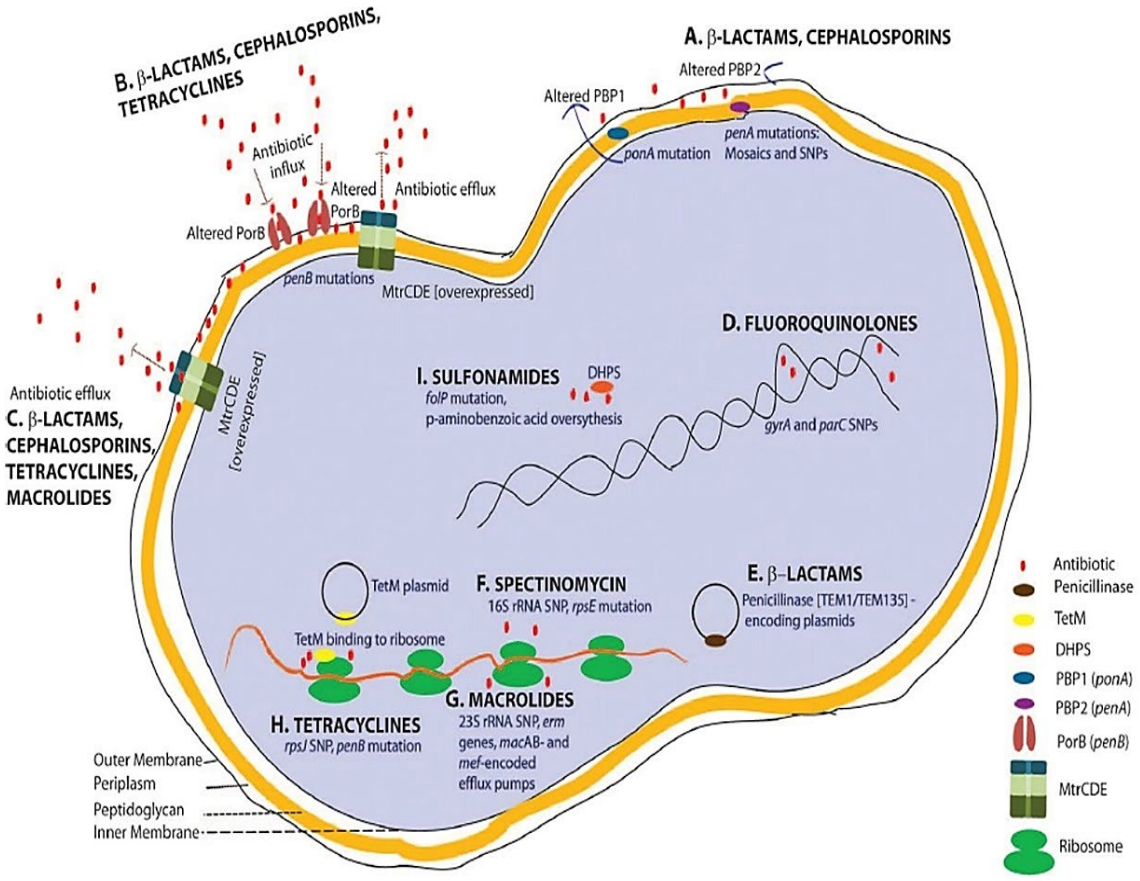

Figure 2.3 Generalised scheme for antimicrobial mechanisms in *Neisseria gonorrhoeae* and the respective antimicrobial drugs. (Source: Dillon et al., 2016)

Neisseria gonorrhoeae mainly acquires the antimicrobial resistance determinants through chromosomally mediated mutations and plasmids carrying resistance determinants (such as the *bla*$_{TEM}$ and *tetM* genes), which encode high-level resistance to penicillin and tetracyclines respectively (Phillips, 1976; Morse *et al.*, 1986). Furthermore, the gonococcus acquires antimicrobial resistance through horizontal gene transfer from other commensal *Neisseria* spp. through transformation or specific mutations (Saika *et al.*, 2001; Furuya *et al.*, 2007). Commensal *Neisseria* species are frequently exposed to antimicrobials and may harbour the antimicrobial resistance genes, which can be transferred to the gonococci through transformation (Unemo and Nicholas, 2012).

2.5.1 Sulphonamide resistance

Sulphonamides inhibit synthesis of folic acid in the gonococcus by acting on the enzyme dihydropteroate synthetase (DHPS) (Johnson and Morse, 1988). Sulphonamide resistance in *N. gonorrhoeae* occurs through either the over production of p-aminobenzoic acid which results in the dilution of the antimicrobial agent or through the production of a mutated enzyme with reduced affinity to sulphonamide (mutations on DHPS) (Lewis, 2010; Shaskolskiy *et al.*, 2016).

2.5.2 Penicillin resistance

Penicillin is a β-lactam antimicrobial and acts by inhibiting cell wall synthesis by binding to the β-lactam-binding pocket of PBP (Shaskolskiy *et al.*, 2016). *Neisseria gonorrhoeae* resistance to penicillin is due to both chromosomal mutations and conjugative plasmids harbouring the *bla*$_{TEM-1}$ or *bla*$_{TEM-135}$ gene which encode TEM-1-type or TEM-135-type β-lactamases (Figure 2.3). The β-lactamases degrade the penicillin by catalysing the hydrolysis of the cyclic amide bond of the penicillin (Muhammad *et al.*, 2014; Costa-Lourenco *et al.*, 2017). Chromosomally mediated resistance in *N. gonorrhoeae* occurs through cumulative mutations, which modify target proteins (penicillin-binding protein 2 gene (*penA*) and penicillin-binding protein 1A/1B (*ponA)* gene) (Ropp *et al.*, 2002), decreased affinity to the penicillin (*por*B) and increased efflux of the penicillin through overexpression of the efflux pump MtrCDE (Unemo *et al.*, 2016; Shaskolskiy *et al.*, 2019).

2.5.3 Tetracycline resistance

Tetracyclines (e.g. doxycycline) are bacteriostatic antimicrobials that inhibit protein synthesis by binding to the 30S ribosomal subunit (Chopra and Roberts, 2001). *Neisseria gonorrhoeae* resistance to tetracyclines can be mediated by the acquisition of the *tetM* conjugative plasmid resulting in high level resistance to tetracyclines (MIC ≥ 16 μg/mL) (Figure 2.3). The *tetM* binds to the ribosomes thereby releasing the tetracycline molecule resulting in protein synthesis (Burdett, 1986; Chopra and Roberts, 2001). Chromosomally mediated resistance in gonococcus is due to multiple mutations; *rpsJ* gene encoding ribosomal protein S10, overexpression of the MtrCDE efflux pump and decreased permeability through the PorB porin (Johnson and Morse, 1988; Hu *et al.*, 2005).

2.5.4 Spectinomycin resistance

Spectinomycin is an aminocyclitol antibiotic which inhibits protein translation through binding the 30S ribosomal subunit. Gonococcal resistance to spectinomycin occurs through single nucleotide polymorphism (SNPs) in the 16S rRNA resulting in high-level spectinomycin resistance (MIC > 1024 μg/mL) (Galimand *et al.*, 2000; Unemo *et al.*, 2013). Furthermore, mutations in the ribosomal protein S5 (Figure 2.3) associated with high-level or low-level spectinomycin resistance (128 μg/mL) have been reported (Ilina *et al.*, 2013).

2.5.5 Fluoroquinolone resistance

Fluoroquinolones are bactericidal antimicrobials that act by inhibiting the DNA gyrase and topoisomerase IV, subsequently blocking bacterial DNA synthesis (Unemo and Shafer, 2014). The DNA gyrase and topoisomerase IV are prokaryotic specific enzymes essential in super-coiling of the bacterial DNA (Shaskolskiy *et al.*, 2016). Gyrase catalyses the negative supercoiling of DNA and is composed of GyrA and GryB subunits; which are encoded by *gyrA* and *gyrB* genes respectively (Jacoby, 2005). *Neisseria gonorrhoeae* resistance to fluoroquinolones occurs through mutations in the *gyrA* gene leading to amino acid changes at codons 91 (S91F) and 95 (D95N/G); encoding an altered GryA subunit of DNA gyrase (Figure 2.4) (Lindbäck *et al.*, 2002). Furthermore, concomitant mutations in the topoisomerase IV encoded by *par*C gene (D86N, S87N, S88P, and E91A/K/G substitutions) are associated with increased resistance to ciprofloxacin (Trees *et al.*, 1999).

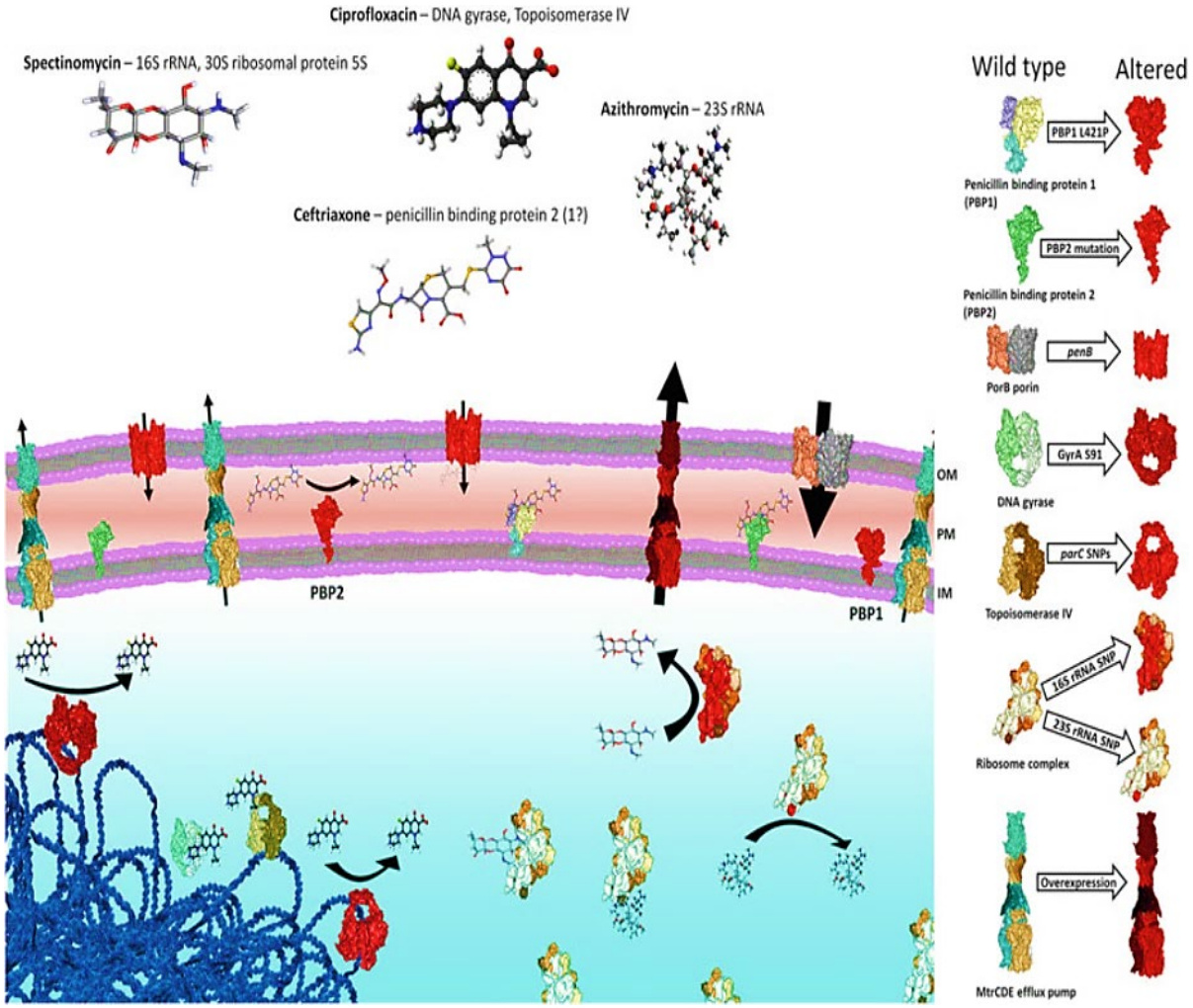

Figure 2.4 Main antimicrobial targets and mechanisms conferring resistance to ciprofloxacin, azithromycin, extended-spectrum cephalosporins and spectinomycin (Source: Unemo et al., 2019a).

2.5.6 Macrolide resistance

Azithromycin is a macrolide antimicrobial which acts by inhibiting protein synthesis by binding to the four alleles of the 23S rRNA component of the 50S ribosomal subunit (Chisholm *et al.*, 2010; Shigemura *et al.*, 2015). Azithromycin is presently recommended for treatment of gonorrhoea in combination with ceftriaxone in South Africa and in many other regions in the world (Department of Health, 2015; Workowski and Bolan, 2015). Azithromycin resistance in *N. gonorrhoeae* has been attributed to three mechanisms: (i) mutations in the peptidyltransferase loop in domain V of the 23S rRNA, (ii) overexpression of the efflux pumps,

and (iii) modification of the ribosomal target by methylase (Unemo and Shafer, 2014; Shigemura *et al.*, 2015).

The 23S rRNA component of the 50S subunit of the bacterial ribosome is the lethal target of macrolide antibiotics. Single nucleotide polymorphisms in 23S rRNA gene encoding peptidyltransferase loop in domain V result in reduced affinity to the 50s ribosomal target for macrolides (Allen *et al.*, 2011; Unemo and Jensen, 2017). Point mutations present in one to four alleles of the 23S rRNA genes are associated to varying azithromycin MICs (Bercot *et al.*, 2014; Costa-Lourenco *et al.*, 2017); substitutions A2059G (*Escherichia coli* numbering) present in three or four alleles of the 23S rRNA *rrl* gene lead to high level azithromycin resistance (MICs ≥ 256 µg/mL) (Chisholm *et al.*, 2010). Moreover, substitutions C2611(*E. coli* numbering) are associated with low to moderate azithromycin resistance (MICs =2 to 32 µg/mL) (Demczuk *et al.*, 2017).

Neisseria gonorrhoeae employs multidrug efflux pumps, which actively expels antimicrobials, as one strategy to avoid action of antimicrobials (Shafer *et al.*, 2016). Five efflux drug pump systems (MtrCDE, MacAB-MtrE, NorM, FarAB-MtrE and MtrF) have been described in the gonococcus, belonging to the resistance nodulation (RND), major facilitator family (MFS), ATP-binding cassette (ABC) and multidrug toxic compound extrusion (MATE) superfamilies (Figure 2.5) (Shafer *et al.*, 2016; Shaskolskiy *et al.*, 2016; Rouquette-Loughlin *et al.*, 2018). The most characterised gonococcus efflux pump is the MtrCDE belonging to the RND family, which exports a wide array of antimicrobials including β-lactams, tetracyclines and macrolides (Hagman *et al.*, 1995; Veal *et al.*, 2002; Rouquette-Loughlin *et al.*, 2018).

The *Cis*- or *trans*-acting regulatory mutations in the MtrCDE efflux pump lead to the overproduction of the *mtrCDE* efflux operon and have been demonstrated to increase the biological fitness of the gonococcus in animal infection models (Warner *et al.*, 2008; Shafer *et al.*, 2016). *Neisseria gonorrhoeae* strains displaying intermediate resistance to azithromycin have missense mutations in the *mtrR* gene causing amino acid substitutions (A39T, G45D, R44H and L47P) (Ilina *et al.*, 2008; Endimiani *et al.*, 2014). Moreover, gonococcal strains displaying high level resistance to azithromycin harbour -35A deletion in the *mtrR* promoter (Figure 2.6) (Lindberg *et al.*, 2007; Unemo and Shafer, 2014).

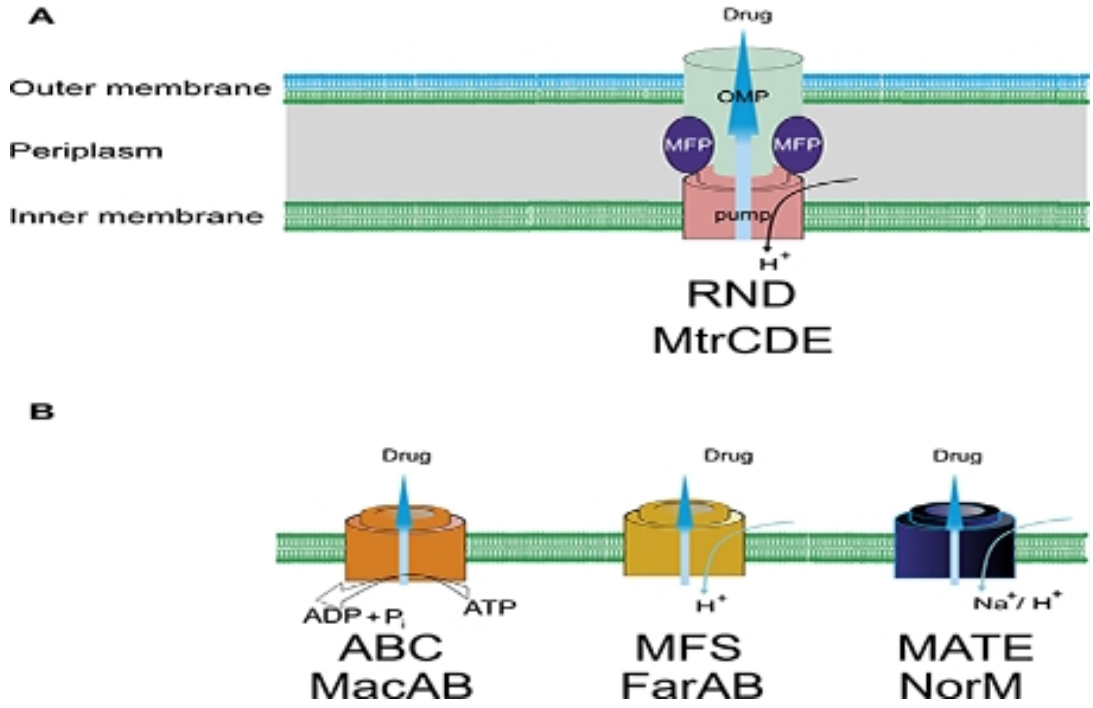

Figure 2.5 Schematic representation of efflux pumps found in *Neisseria gonorrhoeae.* (Source: Shaskolskiy *et al.*, 2016).

Other mutations upstream of the MacAB and NorM efflux pump systems have been shown to confer gonococcal azithromycin resistance through alteration of gene expression (Unemo and Shafer, 2014). Modification of the ribosomal target by methylase or mutations reduces the affinity of the macrolides for ribosomes. *Neisseria gonorrhoeae* strains harbouring the rRNA methylase gene *erm*B has been shown to confer low level azithromycin resistance (Unemo and Shafer, 2014).

2.5.7 Cephalosporin resistance

Cephalosporins are frequently used β-lactam antibiotics for treatment of bacterial infections and have been in use for over 70 years (Page and Badarau, 2008). Extended spectrum cephalosporins, ceftriaxone (injectable) and cefixime (oral) are the most used for treatment of gonorrhoea (Unemo and Shafer, 2014). The primary mechanism of gonococcal resistance to cephalosporins is through mutations that modify the penicillin binding protein (Unemo and Shafer, 2014). *Nesseria gonorrhoeae* has three PBP named PBP1, PBP2 and PBP3 (Dougherty *et al.,* 1980). PBP1 is the major binding site for extended spectrum antibiotics and other beta-lactam antibiotics.

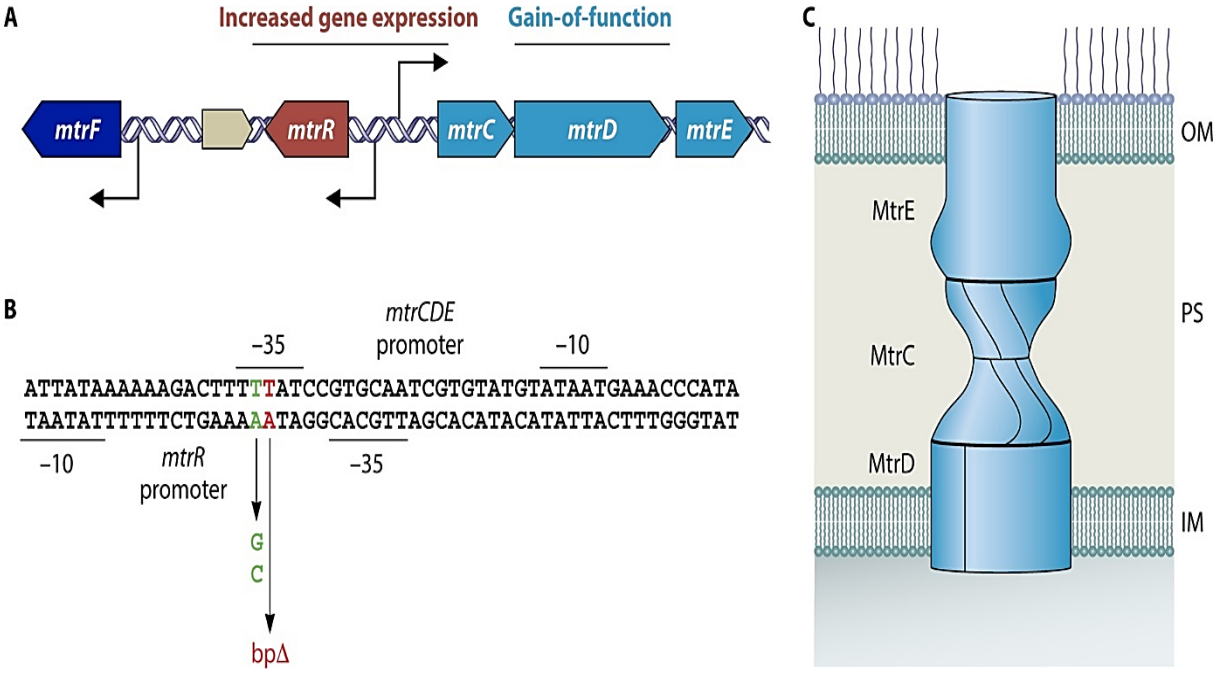

Figure 2.6 Schematic representation of the *mtrR* locus of *Neisseria gonorrhoeae.* (Source: Shafer, 2018).

Reduced gonococcal susceptibility or resistance to extended spectrum cephalosporins is chromosomally mediated, resulting in the alteration of the *penA* gene encoding the PBP-2 or through the insertion of a single amino acid (A501) of a non-mosaic *penA* allele resulting in decreased penicillin binding (Osaka *et al.*, 2008; Barry and Klausner, 2009; Unemo and Shafer, 2014) (Table 2.2). Furthermore, acquisition of a *penA* mosaic alleles encoding altered PBP2 confer resistance of gonococcus to ESCs (Lindberg *et al.*, 2007; Lee *et al.*, 2010). The mosaic *penA* genes are thought to have evolved by acquisition of *penA* gene from the commensal *Neisseria* species (*Neisseria sicca, Neisseria perflava, Neisseria cinerea, Neisseria polysaccharea, Neisseria flavescens*) frequently inhabiting the oropharynx (Lewis, 2010; Unemo and Shafer, 2011). Commensal *Neisseria* species are frequently exposed to antimicrobials and may harbour the antimicrobial resistance genes which can be transferred to the gonococci through transformation (Unemo and Nicholas, 2012). Finally, additional mutations in the promoter and/or coding sequence of the *mtrR* repressor gene cause an overproduction of the MtrCDE drug efflux pump (Hagman *et al.*, 1995), leading to decreased gonococcal susceptibility to extended spectrum cephalosporins (Folster *et al.*, 2009; Zhao *et al.*, 2009). Moreover, specific mutations in the *porB1b* gene (the *penB* resistance determinant) alters the amino acid G101 and A102 of the PorB1b porin (Table 2.2) leading to reduced

susceptibility to extended spectrum cephalosporins (Olesky *et al.*, 2006; Golparian *et al.*, 2010; Ohnishi *et al.*, 2011).

2.6 Detection of antimicrobial resistance in *Neisseria gonorrhoeae*

Neisseria gonorrhoeae culture is the gold standard method for complete antimicrobial resistance testing. Gonococcal antibiotic resistance testing is performed using standardised, qualitative and quality-assured methods such as the Epsilon test (E-test), agar dilution for determining the MIC (mg/L) and the qualitative disc diffusion assay (Unemo, 2015). The disc diffusion method is inferior to the MIC based methods due to the suboptimal correlation to the MIC values and therefore only recommended when agar dilution or E-test are not available particularly in resource-constraint settings (Unemo and Dillon, 2011). The use of nucleic acid amplification tests for diagnosis of gonorrhoea has replaced culture in well-resourced settings due to their excellent sensitivity (Unemo, 2015). Many molecular assays for detection of antimicrobial resistance determinants to penicillins, extended spectrum cephalosporins, fluoroquinolones or multiple drugs have been developed (Low and Unemo, 2016; Donà *et al.*, 2017; Wi *et al.*, 2017).

Table 2.2 Summary of antimicrobial resistance determinants in *Neisseria gonorrhoeae* for antimicrobials used for treatment of gonorrhoea

Antimicrobial	Resistance determinants/mechanisms
Extended-Spectrum cephalosporins	• Mosaic *pen*A alleles: mosaic *pen*A alleles, encoding PBP2 amino acid alterations. • Amino acid alterations: A311V, I312M, V316T, V316P, T483S, A501P, A501V, N512Y, and G545S. • *pen*A SNPs: A501V and A501T alterations in nonmosaic alleles • *pen*A mutations: D345 amino acid insertion in PBP2 and amino acid alterations in the PBP2. • *mtr*R mutations: in promoter (frequently a single A deletion in the 13-bp inverted repeat sequence) or coding sequence (usually a G45D amino acid alteration) • *porB1b* SNPs: G120K and G120D/A121D in PorB1b that decrease influx.
Azithromycin	• 23S rRNA SNPs: C2611T and A2059G, resulting in low-level and high-level resistances, respectively • *mtr*R mutations: in promoter (frequently a single A deletion in the 13-bp inverted repeat sequence) or coding sequence (usually a G45D amino acid alteration) • *erm* genes (*ermB*, *ermC*, and *ermF*): acquired from other bacterial species and encode rRNA methylases that can methylate nucleotides in the 23S rRNA azithromycin target • MacAB efflux pump: overexpression can elevate the MICs of azithromycin • *mef*-encoded efflux pump: acquired from other bacterial species and export macrolides out of the bacterial cell and elevate the MICs of macrolides
Spectinomycin	• 16S rRNA SNP: C1192U substitution • *rpsE* mutations: T24P alteration, V25 deletion, and K26E alteration
Ciprofloxacin	• *gyrA* SNPs: frequently S91F, D95N, and D95G alterations • *parC* SNPs: frequently D86N, S88P, and E91K alterations

Source: (Unemo *et al.*, 2019a)

2.7 Current treatment of gonorrhoea

The WHO recommends a single-dose empiric therapy for gonococcal infection at the first visit of the patient to the healthcare centre (WHO, 2004). Furthermore, the WHO guidelines recommend a therapeutic success of at least 95% for an antimicrobial agent to be used as first-line therapy (Tapsall *et al.*, 2009; WHO, 2012). Consequently, when the prevalence of antimicrobial resistance to a first-line empiric antibiotic exceeds 5% of *N. gonorrhoeae* cases in the general population, or >1% in high frequency transmitters such as MSM and CSW, such treatment should be discontinued (CDC, 1987; WHO, 2004; Unemo *et al.*, 2019b).

In many countries dual antimicrobial therapy consisting of an extended spectrum cephalosporins [ceftriaxone 250 to 500 mg (intramuscularly) or cefixime 400 mg (oral) in combination with azithromycin [1 to 2g] is the mainstay empirical first line therapy (Workowski and Bolan, 2015; Costa-Lourenco *et al.*, 2017; British Association of Sexual Health and HIV, 2019; Unemo *et al.*, 2019a) (Table 2.3). Dual antimicrobial therapy was introduced to prevent development of resistance to extended spectrum cephalosporins (Rice, 2015), and in addition azithromycin has activity against concurrent *C. trachomatis* infections and *M. genitalium* infections (Unemo *et al.*, 2019a). Notably, there is a lack of randomised clinical controlled trials (RCTs) data or strong evidence to support implementation of dual therapy for treatment of *N. gonorrhoeae* and the different doses of azithromycin and ceftriaxone given (Rice, 2015; Lewis, 2019; Unemo *et al.*, 2019a).

South Africa has introduced a syndromic management approach for treatment of STIs through algorithms based on the patient's presenting symptoms in primary healthcare (Garrett *et al.*, 2017). In response to escalation of ciprofloxacin-resistant *N. gonorrhoeae* in South Africa (Lewis and Maruma, 2009), the recommended treatment for uncomplicated urogenital gonococcal infections is dual treatment with ceftriaxone 250 mg (injectable) and azithromycin 1 g (oral) since 2015 (Figure 2.7) (Department of Health, Republic of South Africa, 2015).

Table 2.3 Antimicrobial treatment regimens recommended for uncomplicated gonococcal infection worldwide

Country/Region	Therapy	Alternative Treatment regimens	References
South Africa	CRO 250 mg IM + AZM 1 g PO	Gentamycin 240 mg IM + AZM 2g	(Department of Health, 2015)
Europe (ECDC)	CRO 500 mg IM + AZM 2 g PO	CFX 400 mg PO + AZM 2 g PO	(Unemo *et al.*, 2019a)
USA (CDC)	CRO 250 mg IM + AZM 1 g PO	CFX 400 mg PO + AZM 1 g PO	(CDC, 2012)
WHO	CRO 250 mg IM + AZM 1 g PO	CFX 400 mg PO + AZM 1 g PO	(WHO, 2016)
United Kingdom (BASHH)	CRO 1 g IM OR CIP 500 mg PO	CFX 400mg PO + AZM 2g PO	(BASHH, 2019)
Australia	CRO 500 mg IM + AZM 2 g PO	CFX 400mg PO + AZM 1 g PO	(Unemo *et al.*, 2019a)

AZM, azithromycin; CFX, cefixime; CIP, ciprofloxacin; CRO, ceftriaxone; IM (Intramuscular), P.O. (Per os)

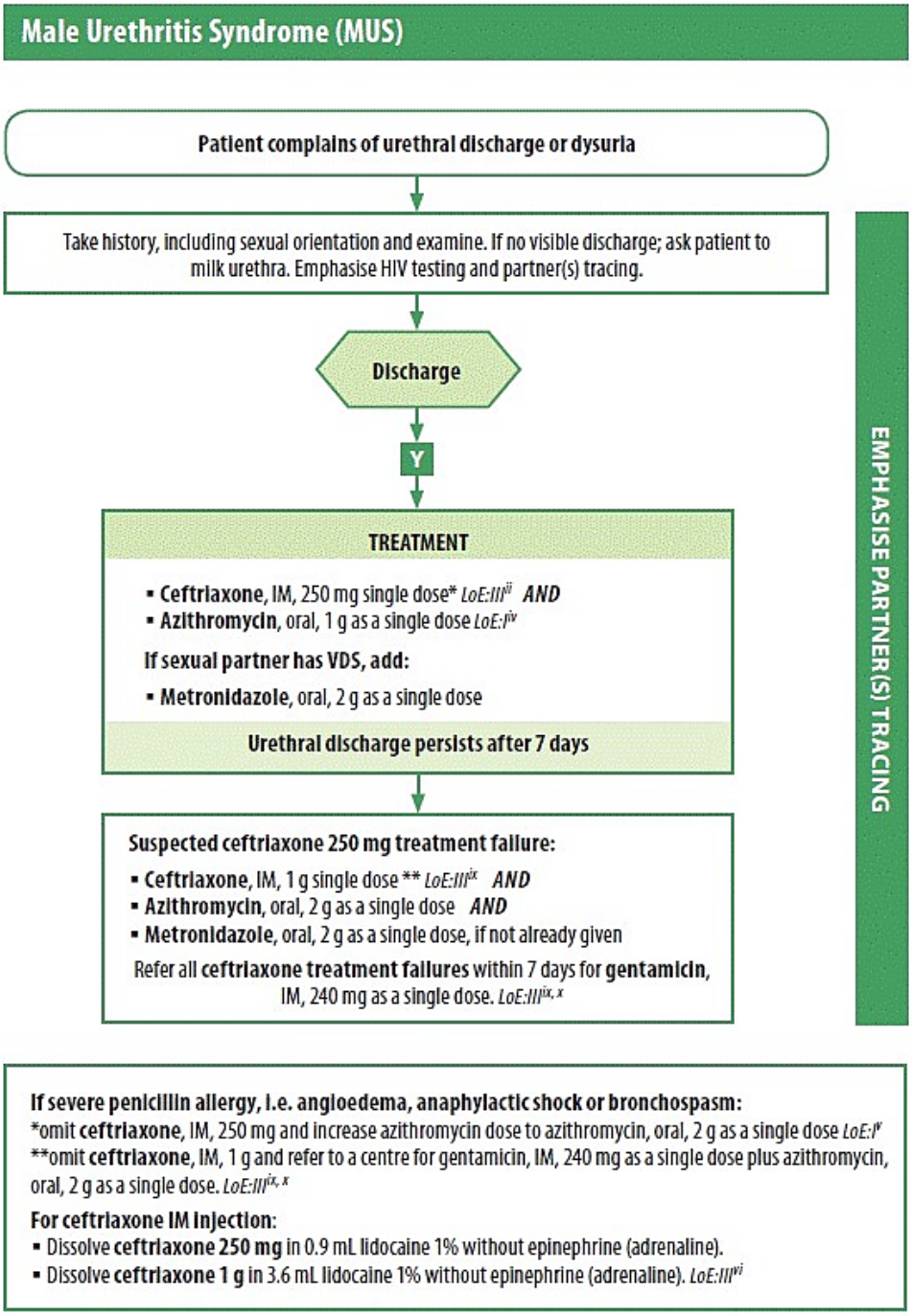

Figure 2.7 Male urethritis syndrome (MUS) treatment flowchart in South Africa (adapted from the Standard Treatment Guidelines and Essential Drugs List) (Source: Department of Health, 2015)

2.8 Future treatment of gonorrhoea

Currently, dual therapy with ceftriaxone plus azithromycin is recommended when local gonococcal antimicrobial resistance data do not support alternate therapeutic options (Unemo, 2015). However, with the emergence of gonococcal strains resistant to ceftriaxone and azithromycin new treatment options are warranted to mitigate the spread of gonococcal resistance (WHO, 2016). Three strategies are currently in pipeline for development of new approaches against antimicrobial resistant *N. gonorrhoeae:* (1) repurposing of old antimicrobial drugs, especially ciprofloxacin, (2) development of novel antimicrobials such as zoliflodacin, and (3) development of alternative therapies such as phage therapy (Suay-García and Pérez-Gracia, 2018).

Due to the lack of alternate antimicrobials to extended spectrum cephalosporins in treatment of gonorrhoea, the use of 'old' antimicrobial drugs which were previously used as first-line drugs has been explored for future treatment of gonorrhoea (Unemo, 2015; Lewis, 2019). Gentamycin which has successfully been used in Malawi for over 20 years for treatment of gonorrhoea (Kamanga *et al.*, 2010), has been re-evaluated in a recent RCT in the UK for use as a dual therapy in combination with azithromycin for treatment of ceftriaxone resistant *N. gonorrhoeae* (Kirkcaldy *et al.*, 2014; Brittain *et al.*, 2016; Ross *et al.*, 2019). However, the results from the clinical trial showed gentamicin was inferior to ceftriaxone; bacteria clearance rate was 91% for the gentamicin arm of the trial compared to 98% in the ceftriaxone arm (Brittain *et al.*, 2016; Ross *et al.*, 2019).

Ciprofloxacin has once again been explored as a potential antibiotic for gonorrhoea therapy in settings where the prevalence of ciprofloxacin resistant *N. gonorrhoeae* is less than 50% and where rapid molecular detection of resistance-conferring genes can be achieved (Lewis, 2019). In the recent UK gonorrhoea treatment guidelines, ciprofloxacin 500 mg (orally) is recommended as the first line antimicrobial if ciprofloxacin antimicrobial susceptibility is known before treatment (Fifer *et al.*, 2019).

On a similar note, modern fluoroquinolones such as sitafloxacin, delafloxacin, gemifloxacin, moxifloxacin and delafloxacin have been evaluated for activity against ciprofloxacin resistant *N. gonorrhoeae* in combination therapy (Soge *et al.*, 2016; Jönsson *et al.*, 2018; Jorgensen *et al.*, 2018). Even though the *in vitro* studies are promising to show excellent activity for these

antimicrobials, further clinical evaluations are required for these antimicrobials to be considered as therapeutic agents of gonorrhoea (Lewis, 2019). Along these lines, Singh *et al*. (2018) recently evaluated 21 dual antimicrobial combinations and identified five potential novel combinations for treatment of resistant *N. gonorrhoeae* infections; namely (1) gentamicin plus cefixime, (2) ertapenem plus gentamicin, (3) ertapenem plus moxifloxacin, (4) ertapenem plus spectinomycin and (5) moxifloxacin plus azithromycin (Singh *et al.*, 2018). However, most of these drugs are relatively expensive and not routinely available in public healthcare sector in many settings.

In response to the emergence of antimicrobial resistance *N. gonorrhoeae* strains, the WHO recently launched a Global Antibiotic Research and Development Partnership (GARDP) in 2017 to guide research and development of new antimicrobials to treat gonorrhoea (Alirol *et al.*, 2017). Currently, solithromycin, zoliflodacin and gepotidacin are novel agents in late stage clinical evaluation for treatment of gonorrhoea (Alirol *et al.*, 2017; Unemo *et al.*, 2017).

Solithromycin (CEM-101) is a broad-spectrum fluoroketolide which inhibits protein synthesis by binding to the 50S ribosomal subunit (Golparian *et al.*, 2012). The phase II trials results were promising, the efficacy was 100% for participants who were culture negative at the test of cure (TOC) (Hook Iii *et al.*, 2015). Subsequently, Phase III trials began, however, the results were disappointing; solithromycin was inferior when compared to the ceftriaxone-based standard-of-care treatment (Chen *et al.*, 2019; Lewis, 2019). In addition, there are safety concerns regarding the risk of hepatotoxicity and hence attempts to proceed with solithromycin for treatment of gonorrhoea have now stalled (Lewis, 2019).

Zoliflodacin (AZD0914/ETX0914) is a first in-class spiropyrimidinetrione and inhibits DNA replication through inhibition of DNA topoisomerase II specifically the *gyr*B and *par*E genes (Huband *et al.*, 2015). Zoliflodacin studies displayed high *in vitro* potency against MDR and XDR *N. gonorrhoeae* isolates from 21 European countries, China and US (Unemo *et al.*, 2015). Moreover, zoliflodacin phase II trial results evaluating the efficacy, tolerability and safety of zoliflodacin, were also promising; the efficacy was 98% to 100% for different doses, the drug was well tolerated and only a minority of the participants reported side effects (Taylor *et al.*, 2018a). In addition, Foerster *et al.* (2019) evaluated zoliflodacin alone or in combination with six antimicrobials (ceftriaxone, cefixime, spectinomycin, gentamicin, tetracycline, cethromycin

or sitafloxacin) for *in vitro* activity against selected WHO reference strains and showed zoliflodacin has potency alone or combination with other antimicrobials against gonococci (Foerster *et al.*, 2019). Accordingly, zoliflodacin will proceed to Phase III RCT in 2019 in several countries including South Africa, The Netherlands, Thailand and USA (Lewis, 2019; Unemo *et al.*, 2019a).

Gepotidacin (GSK2140944) is the first in-class triazaacenaphthylene antimicrobial which inhibits bacterial DNA replication through the inhibition of the DNA topoisomerase II targeting *gyrA* and *par*C genes (Biedenbach *et al.*, 2016). Gepotidacin showed a high potent *in vitro* activity against diverse *N. gonorrhoeae* strains including those with MDR and XDR profiles (Jacobsson *et al.*, 2018). Subsequently, Phase II trials began; gepotidacin achieved >95% microbiological cure rate against treatment of uncomplicated urogenital gonorrhoea (Taylor *et al.*, 2018b). However, participants reported mild to moderate gastrointestinal side effects.

Other non-antimicrobial therapeutic compounds for treatment of gonorrhoea are unfortunately at the early stages of development. These compounds include inhibitors of efflux pumps (e.g pleuro-mutilin) co-administered with relevant antimicrobials, noncytotoxic nanomaterials, innate host immunity, and toxic metabolites (Unemo *et al.*, 2019a). Bacteriophage therapy has recently gained momentum as an alternative therapy for treatment of gonorrhoea due to rising levels of antimicrobial resistant *N. gonorrhoeae* (Suay-García and Pérez-Gracia, 2018). However, phage therapy is in the early stages of development for treatment of gonorrhoea and clinical trials will need to be conducted in future to evaluate the efficacy of this type of treatment (Suay-García and Pérez-Gracia, 2018).

2.9 *Neisseria gonorrhoeae* genotyping

Neisseria gonorrhoeae antimicrobial resistance has emerged, therefore understanding the clonal relationships between resistant *N. gonorrhoeae* strains is an important public health strategy to forestall the evolution and spread of resistant gonococcal strains (Shaskolskiy *et al.*, 2016). Several molecular typing methods based on DNA sequences have been used to characterise gonococcal strains and to track antimicrobial resistant gonorrhoea (Unemo and Dillon, 2011). The DNA sequenced based typing methods compared to other typing methods have a higher resolution, can be used to study molecular epidemiology and are reproducible (Unemo and Dillon, 2011).

2.9.1 *porB* DNA sequence analysis

The major out membrane porin (*porB)*-based DNA sequence analysis is based on sequencing fragments or the entire region of the polymorphic *porB* gene (Bash *et al.*, 2005). The method has been used in various studies describing gonococcal populations since 2000 before the implementation of NG-MAST (Bash *et al.*, 2005). The method is discriminatory and reproducible, and results can be compared between the laboratories (Unemo and Dillon, 2011).

2.9.2 *Neisseria gonorrhoeae* multi-antigen sequence typing

NG-MAST analyses two hypervariable loci of *N. gonorrhoeae; porB* (490 bp) and *tbpB* (390 bp), which encode the B subunit of binding transferrin protein (Martin *et al.*, 2004). This method has a high discriminatory property and has been widely used in micro-epidemiological studies. Moreover NG-MAST is hosted in public database (http://www.ng-mast.net) for analysis of gonococcal DNA sequences and assignment of STs. Recently a new technique for NG-MAST which does not require prior culturing of gonococcal isolates have been developed (Van Der Veer *et al.*, 2018).

2.9.3 *Neisseria gonorrhoeae* sequence typing for antimicrobial resistance

The NG-STAR (https://ngstar.canada.ca) typing scheme was recently developed and utilises DNA sequences of seven known AMR determinants (*penA, mtrR, porB, ponA, gyrA, parC*, and 23S rRNA) associated with resistance to β-lactam antimicrobials, macrolides and fluoroquinolones (Demczuk *et al.*, 2017). The scheme has been used to predict AMR, standardise AMR nomenclature and track gonococcal AMR strains (Demczuk *et al.*, 2017; Eyre *et al.*, 2018).

2.9.4 Multilocus sequence typing

Multilocus sequence typing has been developed for many bacterial pathogens, the scheme examines the alleles of seven housekeeping genes. In *N. gonorrhoeae,* the most widely used scheme uses the sequences of seven housing *Neisseria* species (*abc*Z, *adk*, *aroE*, *fumC*, *gdh*, *pdhC*, and *pgm*), hosted on PubMLST database (http://pubmlst.org/neisseria) (Unemo and Dillon, 2011). However, MLST has several shortcomings such as the suboptimal discriminatory

ability in micro-epidemiological analysis, its high cost and being labour intensive to perform (Unemo and Dillon, 2011).

2.9.5 Whole genome sequencing

The advent of whole genome sequencing has enabled the investigation of gonococcal evolution and transmission of resistance over time (Golparian and Unemo, 2019). Moreover, WGS provides higher resolution compared to other traditional typing methods in describing molecular epidemiology of gonococcal strains, mechanisms of antimicrobial resistance and their emergence and spread (Eyre *et al.*, 2017; Mortimer and Grad, 2018). In combination with the patient metadata, WGS have been used to describe the sexual networks. In a US study, gonococcal lineages associated with cefixime resistance were significantly associated with MSM and transmission was likely to occur from the MSM to the general population (Grad *et al.*, 2014). Furthermore, WGS is compatible with other typing schemes such as NG-MAST, MLST and NG-STAR, while also providing higher resolution and resolve potential transmission links which be inferred by these methods (Didelot *et al.*, 2016; Mortimer and Grad, 2018).

2.10 References

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CHAPTER 3

Genomic analysis of *Neisseria gonorrhoeae* infection among high risk men demonstrates high rates of resistance and a distinct epidemic in South Africa

The editorial style of Sexually Transmitted Infections has been followed for this chapter.

Abstract

Background: *Neisseria gonorrhoeae* antibiotic resistance has emerged worldwide, however, the situation in Sub-Saharan Africa is not well-documented. We investigated the molecular epidemiology and occurrence of antimicrobial resistance in *N. gonorrhoeae* infections in two core transmission groups of men in Johannesburg, South Africa.

Methods. Men who have sex with men (MSM) presenting with urethral discharge and men with a recurrent episode of urethral discharge were recruited. Molecular testing and culture for *N. gonorrhoeae* followed by antimicrobial susceptibility testing was performed. Whole genome sequencing (WGS) was used to identify resistance conferring mutations and to determine genetic relatedness of the isolates.

Results. Fifty-one men were recruited; 42 (82%) had *N. gonorrhoeae* infection. Most gonococcal isolates were resistant to ciprofloxacin (78%) and tetracycline (74%); 33% were penicillin resistant. All gonococcal isolates were susceptible to cephalosporins and spectinomycin. Azithromycin resistance was observed in four (15%) isolates (epidemiological cut-off); all with mutations in the *mtrR* promoter region. WGS revealed a diverse epidemic with mostly novel NG-STAR (70%) and NG-MAST (70%) sequence types.

Conclusions. A high prevalence of antimicrobial-resistance in *N. gonorrhoeae* strains obtained from high-risk men in South Africa was demonstrated. Introduction of diagnostics and scale-up of surveillance are warranted to prevent emergence of multidrug-resistant infections.

Keywords. *Neisseria gonorrhoeae*, core transmission groups, whole genome sequencing, antimicrobial resistance, azithromycin, ceftriaxone, ciprofloxacin

3.1 Introduction

Gonorrhoea is a major public health concern globally; the World Health Organization (WHO) estimated that 87 million new infections occurred among 15-49-year olds in 2016,[1] with the highest incidence found in Sub-Saharan Africa.[2] *Neisseria gonorrhoeae* is included in the WHO global priority list of antibiotic-resistant bacteria as it has developed resistance to every antimicrobial recommended for treatment since the introduction of the sulphonamides in the 1930s.[3 4]

The recent emergence of gonococcal strains in Australia and United Kingdom resistant to ceftriaxone and azithromycin, has raised major concerns of untreatable of gonorrhoea.[5] However, there are limited data available on resistance of *N. gonorrhoeae* strains circulating in Sub-Saharan Africa, the region with the highest burden of infection, and their mechanisms of antimicrobial resistance.[2,6] This paucity of data is due to the lack of access to laboratory diagnostic services and the use of syndromic management for treatment of sexually transmitted infections (STIs) in this region.[7] Syndromic management has many limitations including lack of susceptibility testing, inability to identify asymptomatic infections and limited opportunity for widespread surveillance; data on individuals presenting with treatment failure are not recorded.[8,9] Current syndromic management in South Africa constitutes of 1 g azithromycin with 250 mg ceftriaxone.[10]

Core transmission groups such as men who have sex with men (MSM) and those with recurrent episodes of gonorrhoea have played an important role in the emergence of drug resistance.[11] In South Africa[12,13] and elsewhere in Africa, there is a high burden of *N. gonorrhoeae* infections among MSM[14,15]; the first two cases of cefixime-resistant *N. gonorrhoeae* infection in Africa were reported in this population.[16] It is therefore imperative to understand the antibiotic resistance profile of gonococcal populations in core transmission groups, and to include these populations in sentinel surveillance to inform clinical management guidelines and policy design.[17]

Whole genome sequencing (WGS) provides a high resolution molecular epidemiological tool for describing gonococcal populations and their mechanisms of antimicrobial resistance.[18,19] However, conducting quality-assured WGS studies of *N. gonorrhoeae* in low-resource settings may be challenging and is rarely conducted due to poor laboratory infrastructure and the

prolonged time required for specimens to reach the laboratory for culture.[7,20] Also, surveillance systems are limited due to a lack of resources; generally these systems do not include core transmission groups as these are hard to reach due to cultural issues.[7,20] Nevertheless, molecular epidemiological studies are essential to map the status of antimicrobial resistance in *N. gonorrhoeae* infections in sub-Saharan Africa and to monitor for emergence of resistance.

A cross-sectional study was conducted to assess the antimicrobial resistance profiles and to determine the molecular epidemiology of *N. gonorrhoeae* in two core groups of high-risk men visiting sexual health services in Johannesburg, South Africa

3.2 Methods

3.2.1 Study Design and Setting

This cross-sectional study was conducted at three primary healthcare facilities (PHC) offering MSM friendly health services to men in Johannesburg, South Africa, between March 2018 and April 2019. One facility is in the city centre and the other two are in Soweto, Johannesburg's largest township. Most of the clients visiting these facilities are of low socio-economic status.[21] The study was approved by the Faculty of Health Sciences Research Ethics Committee of the University of Pretoria, South Africa (Ref 253/2017) and the Johannesburg District Research Committee. Written informed consent was obtained from all participants.

3.2.2 Study population

Two groups of men (>18 years old) at high risk of *N. gonorrhoeae* infections in the study were recruited: (1) men with recurrent or persistent discharge within one month of initial treatment as provided by a healthcare facility in the public or the private sector in South Africa, and (2) MSM (born male with any type of sexual contact with another men in the preceding six months) presenting with urethral discharge.

3.2.3 Study Procedures

Physical examination was conducted, and questionnaires were administered in a face-to-face interview to collect data on demographics and recent sexual behaviour. Study nurses collected urethral swabs (Copan Diagnostics, Italy), which were immediately inoculated on New York

City (NYC) agar (Thermo Scientific, Lenexa, USA) medium at the study site followed by transport in an AnaeroPack^{TM} (Thermo Scientific, Lenexa, USA) to the Department of Medical Microbiology at the University of Pretoria for further processing. First-void urine was collected for molecular detection of *N. gonorrhoeae, C. trachomatis*, *T. vaginalis* and *M. genitalium* infection. Participants were provided with standard syndromic management upon recruitment [Ceftriaxone 250 mg (IM) and azithromycin 1 g (PO)]; follow-up treatment was provided based on laboratory results if indicated.[10]

3.2.4 Microscopy, culture and antimicrobial susceptibility testing

The inoculated NYC agar plates were incubated at 37°C with 5% CO_2 at the Department of Medical Microbiology, University of Pretoria, and were assessed for microbial growth daily. Presumptive *N. gonorrhoeae* colonies on NYC agar plates were identified using Gram stain, rapid oxidase test and API NH (bioMérieux, France). This was followed by antimicrobial susceptibility testing to ciprofloxacin, azithromycin, ceftriaxone, cefixime, penicillin G, tetracycline and spectinomycin using E-tests (bioMérieux, France) following manufacturer's instructions. Minimum inhibitory concentrations (MICs) were interpreted according to European Committee of Antimicrobial Susceptibility Testing breakpoints (EUCAST), except for azithromycin where the epidemiological cut-off (ECOFF = 1 mg/L) value was used as no resistance breakpoint exists (www.eucast.org). The *N. gonorrhoeae* ATCC 49266 and 19424 strains were used as quality control.

3.2.5 Molecular detection of pathogens causing male urethritis syndrome

Urine specimens were tested for presence of *N. gonorrhoeae* and *C. trachomatis* DNA using Lightmix 480 HT CT/NG assay (TIB MOLBIOL, Berlin, Germany). The presence of *M. genitalium* and *T. vaginalis* DNA were tested for with validated in-house real time polymerase chain reaction (PCR) as described elsewhere.[22,23] Additionally, *C. trachomatis* positive samples were tested for the lymphogranuloma venereum (LGV) biovar as previously described [24] and *M. genitalium* positive specimens were assessed for presence of macrolide resistance.[25]

3.2.6 DNA preparation and whole genome sequencing

Genomic DNA was prepared from single colonies using High Pure PCR Template Preparation kit (Roche, Germany). The *N. gonorrhoeae* DNA sequencing library was prepared using the NexteraXT library preparation kit (Illumina, Eindhoven, the Netherlands). Paired-end 250-bp indexed reads were generated on the Illumina MiSeq instrument following manufacturer's instructions (Illumina, Eindhoven, the Netherlands). In brief, raw reads were assessed for quality using FastQC[26], low-quality bases and adaptor sequences were trimmed with Trimmomatic.[27] Contaminants were investigated using Kraken v2.0.7 and removed using DeconSeq v.4.3.[28,29] Raw reads were *de novo* assembled using SPAdes v.3.9.0 [30] and quality was assessed using Quast v4.3.[31] WGS read data was submitted to National Centre for Biotechnology Information under BioProject accession No PRJNA575338.

3.2.7 Molecular epidemiological analysis

Core genome single nucleotide polymorphisms (SNPs) were determined using ParSNP v1.2[32] using the FA1090 reference strain (GenBank accession number NC_002946.2). Recombination blocks were removed using Gubbins v1.4.10[33] and maximum-likelihood phylogenetic tree was generated with RAxML v8.2.9 with GTRCAT substitution model[34]. Phylogenetic trees were visualized and annotated using Interactive Tree of Life.[35]

For analysis of genes implicated in drug resistance, we used the *N. gonorrhoeae* sequence typing for antimicrobial resistance (NG-STAR) database, which utilises DNA sequences of seven known AMR determinants (*penA, mtrR, porB, ponA, gyrA, parC* and 23S rRNA)[36] and the Bacterial Isolate Genome Sequence (BIGSdb) genomics platform (https://pubmlst.org/neisseria).[37] New alleles and allele combinations were submitted to https://ngstar.canada.ca for assignment of new sequence types (STs).

To determine molecular epidemiology of the isolates, NG-MAST (http://www.ng-mast.net) and multilocus sequence typing (MLST) (https://pubmlst.org/neisseria/) databases were used *in silico* from WGS data to assign allele numbers and STs. Any novel STs were submitted to the curator for number assignment. NG-MAST STs which were yet to be assigned STs were inferred manually using a unique number. In case of positive molecular results for *N.*

gonorrhoeae but no growth in culture, culture-free NG-MAST was performed on non-culture viable PCR positive *N. gonorrhoeae* clinical samples as previously described.[38]

3.2.8 Statistical analysis

Descriptive data are provided as number with proportion and median with range. Comparison of categorical variables between groups was done using the Chi-squared test with Fisher's Exact test when appropriate.

3.3 Results

3.3.1 Characteristics of the Study Population

A total of 51 men were recruited in the study; 31 were from the two township clinics and 20 from the inner-city clinic. The median age was 27 years (range 22-38 years) and seven men (14%) were HIV-infected of whom six (86%) men were on antiretroviral therapy **(Table 3.1)**. More than half of the men (n=31; 61%) reported that their discharge was recurrent or persistent following recent treatment. At enrolment, 21 (41%) men identified as homosexual, 11 (22%) bisexual and 19 (37%) heterosexual. Overall, most participants 44 (86%) reported multiple sex partners in the previous six months and three-quarters of them [38 (75%)] had been treated for an STI in the previous six months.

3.3.2 Aetiology of male urethritis syndrome at enrolment

An STI was detected in urine specimens of 90% (46/51) of men: 82% (42/51) participants had *N. gonorrhoeae* infection followed by *C. trachomatis* (11/51; 22%), *M. genitalium* (10/51; 20%) and *T. vaginalis* (4/51; 8%) **(Table 3.2)**. Twenty-one men (41%) had more than one STI detected. *N. gonorrhoeae* was more commonly detected among MSM (n=29/32; 91%) compared to heterosexual men (n=13/19; 68%) with recurrent or persistent discharge ($p<0.05$). All 11 *C. trachomatis* positive specimens tested negative for the LGV biovar. *M. genitalium* macrolide resistance associated mutation in the 23S rRNA (A2071G) and a ParC mutation (Pro-62→Ser) associated with quinolone resistance were detected in one case.[39]

3.3.3 Antimicrobial susceptibility testing

Gonococcal cultures were positive for 27/42 men (64%) with *N. gonorrhoeae* detected molecularly. Eight (30%) of the cultured isolates demonstrated phenotypical resistance to three or more antimicrobial classes and could be classified as multidrug-resistant (MDR) *N. gonorrhoeae*.[40] Antimicrobial susceptibility data and molecular markers associated with resistance of *N. gonorrhoeae* are presented in **Tables 3.1 and 4.1**

Most of the isolates were phenotypically resistant to ciprofloxacin (n=21; 78%). The following mutations in the GyrA were identified: 21 (100%) Ser-91→Phe, 13 (62%) Asp-95→Gly and 8

(38%) Asp-95→Ala. ParC mutations were observed in 16 isolates of which 15 were ciprofloxacin resistant based on phenotypic testing.

Azithromycin resistance interpreted using ECOFF, was identified in 4/27 (15%) gonococcal isolates (MIC range 1 to 8 µg/mL); all had been obtained from MSM. The *mtrR* promoter adenine deletion (-35A del) was identified in two isolates (MICs 4 and 8 µg/mL) and an Ala-39→Thr mutation in the MtrR repressor was identified in the other two (MIC of 1 µg/mL). An Ala-39→Thr mutation was identified in 6/27 azithromycin isolates classified susceptible as per ECOFF (MIC range 0.5-0.75 µg/mL). The azithromycin MIC distributions are shown **Figure 3.1**. No known mutations in the 23S rRNA and *macAB* promoter that confer resistance to macrolides were detected.

There was no resistance or decreased susceptibility to cefixime or ceftriaxone among our isolates, but one-third of isolates (9/27; 33%) demonstrated resistance to penicillin. All resistant isolates had the Leu-421→Pro mutation in the penicillin-binding protein (PBP1) associated with penicillin resistance.[41] The following PorB1b mutations were also identified: Gly-120→Lys and Ala-121→Asp (1/9), Gly-120→Asn and Ala-121→Asn (1/9), and Ala-121→Ser (1/9). Furthermore, 4/9 isolates had the Ala-39→Thr mutation in the MtrR repressor associated with overexpression of MtrCDE efflux pump. An adenine deletion (-35A del) in the promoter region of *mtrR* was identified in one gonococcal isolate. Eight out of nine penicillin resistant isolates harboured the $bla_{\text{TEM-1}}$ plasmid; the ninth had $bla_{\text{TEM-135}}$ (MIC= 12µg/mL) associated with resistance to β-lactams.[42]

Most of the isolates, 20/27 (74%) were phenotypically resistant to tetracycline. All tetracycline-resistant isolates harboured a conjugative plasmid *tetM*: 15 (75%) were of American and five (25%) of the Dutch type. Five isolates demonstrated high level plasmid-mediated tetracycline resistance (MIC ≥ 16 µg/mL); all harboured American-type *tetM* plasmid. Two (10%) isolates had the -35A del in *mtrR* promoter, and 10 isolates (50%) had the Ala-39→Thr mutation, whereas a Val-57→Met mutation in the ribosomal protein S10 (*rpsJ*) was detected in 14 (70%) of the 20 tetracycline-resistant gonococcal isolates.[43] No resistance to spectinomycin was observed.

Table 3.1 Demographic characteristics of the study population (n=51)

Characteristics	MSM with urethral discharge (n= 20)	MSM repeat STI symptoms (n= 12)	Heterosexual men with repeat STI symptoms (n=19)
Employment status			
Self-employed	1 (5)	0 (0	2 (11)
Formal employment	12 (60)	5 (42)	9 (47)
Unemployed	6 (30)	4 (33)	8 (42)
Student	1 (5)	3 (25)	0 (0)
Sexual orientation			
Heterosexual	0 (0)	0 (0)	19 (100)
Homosexual	15 (75)	6 (50)	0 (0)
Bisexual*	5 (25)	6 (50)	0 (0)
Sexual practice in last 6 months			
Sex with men only	15 (75)	6 (50)	0 (0)
Sex with females only	0 (0)	0 (0)	19 (50)
Sex with both men and women	5 (25)	6 (50)	0 (0)
Condom use last sex act			
Yes	4 (20)	1 (8)	2 (11)
No	16 (80)	11 (92)	17 (89)
Multiple sex partners in last 6 months			
Yes	15 (75)	10 (83)	19 (100)
No	5 (25)	2 (17)	0 (0)
Treated for any STI-associated symptoms in the past 6 months			
Yes	7 (35)	12 (100)	19 (100)
No	13 (65)	0 (0)	0 (0)
HIV status at enrolment			
Positive	2 (10)	4 (33)	1 (5)
Negative	18 (90)	8 (67)	16 (84)
Unknown	0 (0)	0 (0)	2 (11)
Reporting PrEP use			
Yes	8 (40)	1 (8)	0 (0)
No	12 (60)	11 (92)	0 (0)

Data are presented as numbers with proportion.

* Bisexual men counted as MSM

Abbreviations: MSM, men who have sex with men; STI, sexually transmitted infection; HIV, Human immunodeficiency virus, PrEP, pre-exposure prophylaxis

Table 3.2 Sexually transmitted infections detected from study participants with male urethritis syndrome in Johannesburg, South Africa (n=51)

Microorganism	Men who have sex with men (n=32)[a]	Heterosexual men (n=19)[b]
Neisseria gonorrhoeae	29 (91)	13 (68)[c]
Chlamydia trachomatis	7 (22)	3 (16)
Mycoplasma genitalium	5 (16)	4 (21)
Trichomonas vaginalis	4 (13)	3 (16)

Data are presented as numbers with proportion.

[a]13 men had a mixed infection detected: *Neisseria gonorrhoeae* and *Chlamydia trachomatis* (n=7), *Neisseria gonorrhoeae* and *Mycoplasma genitalium* (n=2), and *Neisseria gonorrhoeae* and *Trichomonas vaginalis* (n=2).

[b]8 men had a mixed infection detected: *Neisseria gonorrhoeae* and *Chlamydia trachomatis* (n=2), *Neisseria gonorrhoeae* and *Mycoplasma genitalium* (n=4), and *Neisseria gonorrhoeae* and *Trichomonas vaginalis* (n=3).

[c]*Neisseria gonorrhoeae* was identified as aetiological agent of discharge more frequently in men who have sex with men compared to heterosexual men (Fisher's Exact Test, p=0.04).

Table 3.3 Antimicrobial susceptibility profiles and minimum inhibitory concentrations of *Neisseria gonorrhoeae* isolates collected from men in South Africa (n=27)

Drug	Susceptible* n (%)	Resistant n (%)	Median MIC value	MIC range (µg/mL)
Ciprofloxacin	6 (22)	21 (78)	0.75	0.002-4
Azithromycin**	23 (65)	4 (15)	0.25	0.023-8
Penicillin	18 (67)	9 (33)	0.5	0.016-12
Tetracycline	7 (26)	20 (74)	8	0.125-96
Spectinomycin	27 (100)	0	6	4-48
Cefixime	27 (100)	0	0.016	0.016-0.023
Ceftriaxone	27 (100)	0	0.002	0.002-0.004

Abbreviation: MIC, minimum inhibitory concentration.

*EUCAST breakpoints were used to classify strains as susceptible or resistant.

*no EUCAST resistance breakpoint exists and instead the epidemiological cut-off (ECOFF) value of 1.0 µg/mL was used for interpretation.

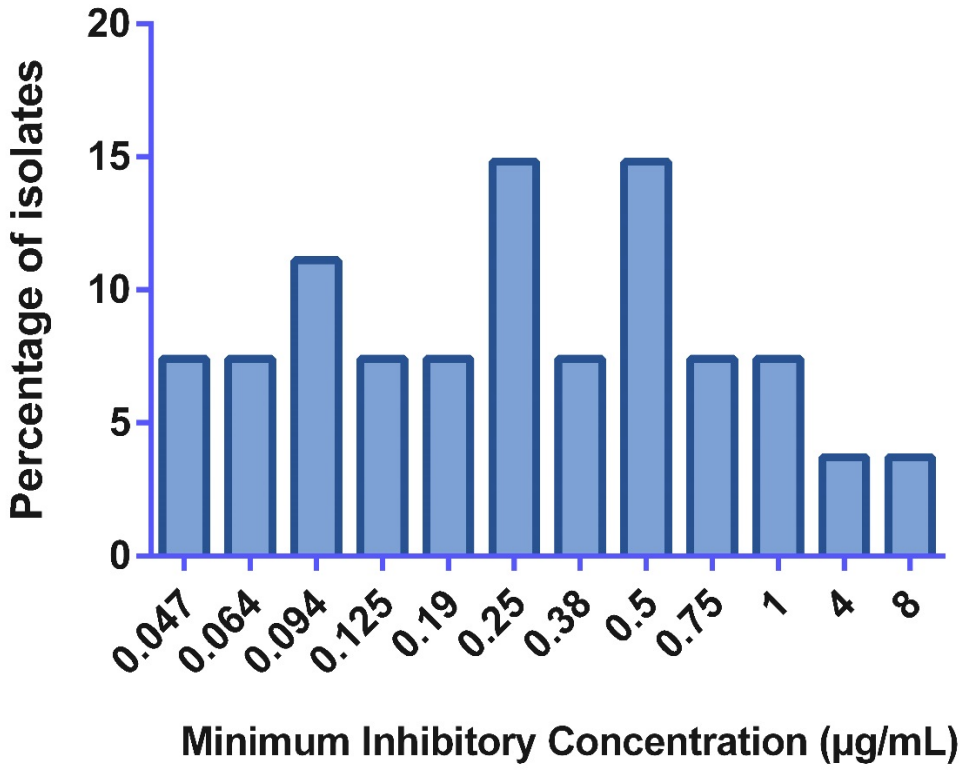

Figure 3.1 Minimum inhibitory distributions for azithromycin in *Neisseria gonorrhoeae* isolates from men in South Africa

3.3.4 Molecular epidemiology and phylogenetic analysis

The phylogenetic analysis of WGS data of the 27 gonococcal isolates revealed a large diversity among *N. gonorrhoeae* strains. The gonococcal strains were interspersed across the phylogenetic tree; no clustering based on the location where the isolates were collected, sexual orientation of the participants or antimicrobial resistance profiles were observed (**Figure 3.2**). The NG-STAR analysis identified 24 different STs; 19 (70%) were novel (**Table 3.5**). Five strains with novel NG-STAR STs had novel *mtrR* alleles, whereas NG-STAR ST1942, ST1935 and ST1930 possessed novel *parC* alleles.

Table 3.4 Resistance-associated mutations identified in *Neisseria gonorrhoeae* isolates resistant to penicillin, ciprofloxacin, tetracycline, azithromycin and tetracycline

Drug	Resistant isolates (n)	Isolates with molecular resistance determinants (n)	Protein	Mutation	Isolates with this gene/ mutation n (%)
Ciprofloxacin	21	21	GyrA	Ser-91→Phe	21 (100)
				Asp-95→Gly	13 (62)
				Asp-95→Ala	8 (38)
			ParC	Ser-87→Ile	2 (10)
				Ser-87→Asn	6 (29)
				Glu-91→Gly	1(5)
				Asp-86→Asn	6 (29)
Azithromycin	4	4	*mtrR*-promoter	-35A del	2 (50)
			MtrR	Ala-39→Thr	2 (50)
Penicillin	9	9	PonA	Leu-421→Pro	9 (100)
			PorB1b	Gly-120→Lys & Ala-121→Asp	1(11)
				Gly-120→Asn & Ala-121→Asn	1 (11)
				Ala-121→Ser	1 (11)
			mtrR-promoter	-35A del	1 (11)
			MtrR	Ala-39→Thr	4 (44)
			$bla_{\text{TEM-1B}}$	-	8 (89)
			$bla_{\text{TEM-135}}$	-	1 (11)
Tetracycline	20	20	PorB	Gly-120→Lys & Ala-121→Asp	2 (10)
				Gly-120→Asn & Ala-121→Asn	1 (5)
				Ala-121→Ser	6 (30)
			mtrR promoter	-35A del	2 (10)
			MtrR	Ala-39→Thr	10 (50)
			*tet*M (Dutch)	-	5 (25)
			*tet*M (American)	-	15 (75)
			RpsJ	Val-57→Met	14 (70)

The MLST typing identified 18 different STs of which five were new to the PubMLST database. The most frequently identified sequences types were ST1588 (n=3), ST1893 (n=3), ST13942 (n=3) and ST1579 (n=2). *In silico* analysis of WGS data classified 27 isolates into 24 different NG-MAST STs. Most of these STs (n=19; 70%) have not been previously described; we identified seven novel *porB* and nine novel *tbpB* alleles. We further employed culture-free NG-MAST on the 15 *N. gonorrhoeae* PCR positive specimens without growth in culture. Ten (67%) clinical samples were successfully genotyped resulting in both *porB* and *tbpB* sequences and these were allocated an NG-MAST ST; for five specimens only the *porB* but not *tbpB* results were generated. The NG-MAST dendrogram (**Figure 3.3**) confirms the genetic diversity observed using WGS and culture-free NG-MAST.

Table 3.5 The NG-STAR profiles of *Neisseria gonorrhoeae* strains isolated from high-risk men in Johannesburg, South Africa (n=27)

Isolate number	*penA*	*mtrR*	*porB*	*ponA*	*gyrA*	*parC*	23S rRNA	NG-STAR ST
JR-401	19.001	278[a]	100	100	100	103[a]	100	1942*
JR-402	19.001	10	14	1	7	26	100	1931*
JR-403	9.001	69	14	1	1	22	22	1940*
JR-404	2.002	18	14	1	1	4	100	1934*
JR-405	19.001	18	3	1	1	4	100	1937*
JR-406	19.001	18	3	1	7	102[a]	100	1935*
JR-407	9.001	69	19	1	1	22	100	1623
JM-510	9.001	69	19	1	1	22	100	1623
JR-408	19.001	18	100	1	7	15	100	1632
JR-409	9.001	10	23	1	1	55	100	1932*
JR-410	19.001	279[a]	24	1	7	15	100	1945*
JR-411	1.001	54	14	1	7	26	100	1939*
JR-412	2.002	10	3	100	1	89	100	1929*
JR-413	14.001	277[a]	41	100	1	4	100	1944*
JR-414	2.002	40	100	100	7	4	100	427
JR-415	2.002	171	13	100	1	90	100	1941*
JM-501	14.001	130	3	100	100	2	100	520
JM-502	2.002	69	100	100	100	2	100	1835
JM-503	2.002	40	3	100	7	4	100	1938*
JM-504	9.001	276[a]	14	1	1	49	100	1943*
JM-509	9.001	276[a]	14	1	1	49	100	1943*
JM-505	2.002	280[a]	14	1	1	4	100	1946*
JM-506	9.001	1	8	1	100	2	100	139
JM-508	9.001	1	8	1	100	2	100	139
JM-507	19.001	10	100	1	7	26	100	1933*
JM-511	19.001	18	100	1	100	89	100	1936*
JM-512	2.002	10	3	100	1	102[a]	100	1930*

Novel NG-STAR Sequence Types that were identified are indicated with an asterix (*)

[a] Novel NG-STAR alleles as identified in this study

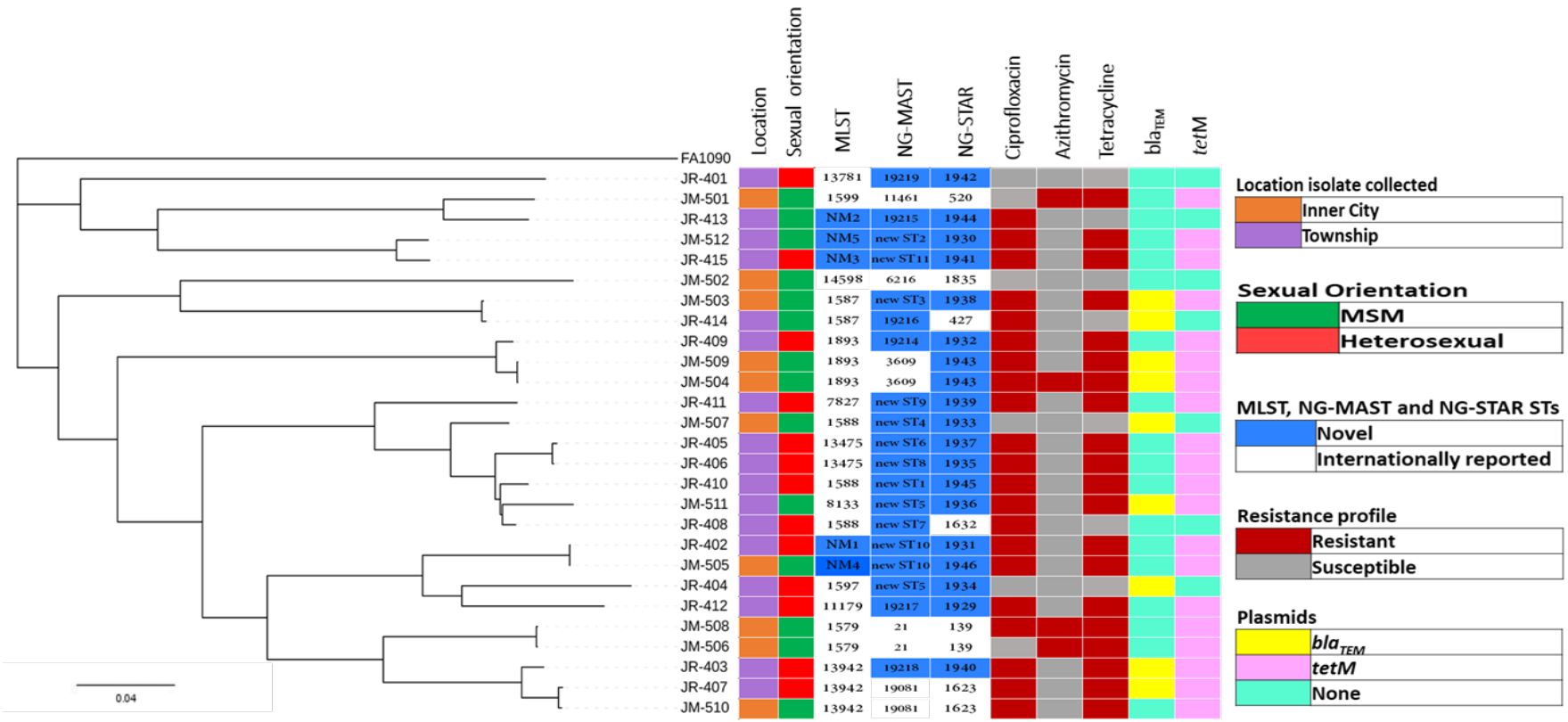

Figure 3.2 Whole-genome core single-nucleotide polymorphism maximum likelihood phylogenetic tree of 27 *Neisseria gonorrhoeae* strains obtained in South Africa including the FA1090 reference strain. The length scale bar represents the estimated evolutionary divergence between isolates. Location where isolates were obtained, sexual orientation, multilocus sequence type (MLST), multiantigen sequence type (NG-MAST) and *N. gonorrhoeae* sequence typing for antimicrobial resistance (NG-STAR) are indicated. Susceptibility to ciprofloxacin (CIP), azithromycin (AZI) and tetracycline (TET) resistance shown, bla_{TEM} gene (light yellow) and *tetM* gene (pink) on plasmids. Novel sequence types are highlighted in blue.

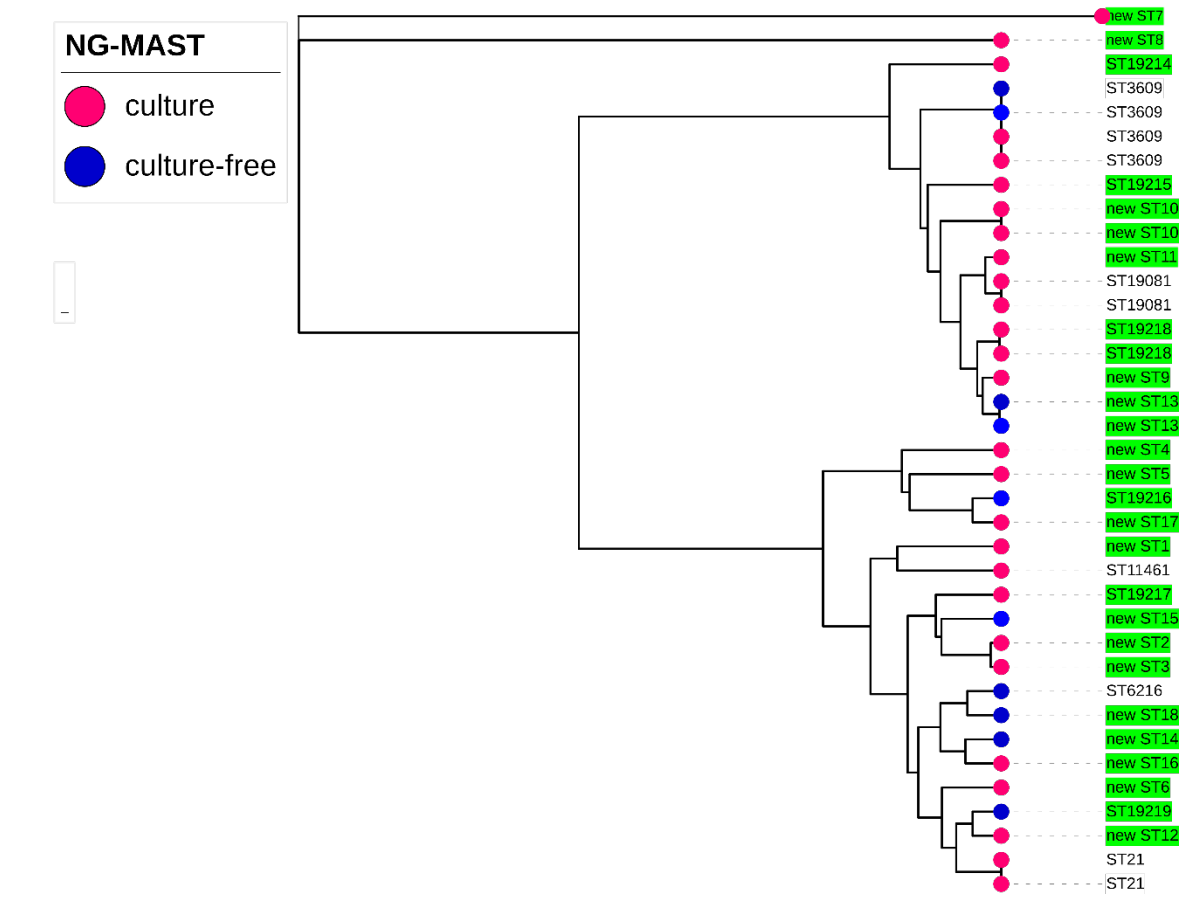

Figure 3.3 Dendrogram constructed by multiple alignment of concatenated *porB* and *tbpB* sequences clustered with unweighted pair group method with arithmetic mean (UPGMA) algorithm. New NG-MAST sequence types identified in this study are highlighted in green.

3.4 Discussion

This study is among the first in Africa to provide a comprehensive in-depth WGS analysis of *N. gonorrhoeae* infection among core transmission groups that are generally associated with emergence of antimicrobial resistance. We have demonstrated that urethral gonococcal infection and antimicrobial resistance are highly prevalent in this selected population of high-risk men in South Africa. We also demonstrate that, in this population, there is a distinct epidemic of *N. gonorrhoeae* strains circulating that is genetically unique based on the high number of novel STs identified.

As expected from other studies, *N. gonorrhoeae* infection was identified as the main aetiological cause of urethral discharge in men in our study.[12,13] Most participants reported unprotected sex and multiple sex partners; the coinfection rate with other STIs was high confirming that we recruited a core transmission group of high-risk men.[12,43,44] Although our study cohort may not be fully representative of the general male population, it constitutes an important sentinel population to study the emergence of *N. gonorrhoeae* resistance and the potential for epidemic spread.[4] One-third of the isolated *N. gonorrhoeae* strains in our study was classified as MDR and high rates of ciprofloxacin and tetracycline resistance were observed; these drugs were both used in syndromic management of STIs for a prolonged period of time in South Africa; ciprofloxacin was only discontinued in 2008 and doxycycline in 2015.[45] These high rates of antimicrobial resistance for these two drugs are in line with reports from national surveillance and another recent study conducted in the KwaZulu-Natal province in South Africa.[45 46] The cumulative mutations in the quinolone resistance determining regions of GyrA and ParC that we report have been previously described.[47]

Although ciprofloxacin was discontinued more than a decade ago resistance rates remain high.[45] Possible explanations for this are the use ciprofloxacin in management of dysuria in men and as, in our experience, continued use of ciprofloxacin for treatment of male urethral discharge by some of the private practitioners in our area. The continuous high resistance rate means that repurposing of this drug for syndromic management is not feasible at this stage. The high rate of tetracycline resistance that we observed could be attributed to the use of doxycycline in syndromic management

of non-gonococcal urethritis until five years ago in South Africa.[10] Most of the tetracycline-resistant isolates harboured the well-known *tetM* plasmid and RpsJ V57M mutations.[48]

Azithromycin was only introduced in 2015 in the syndromic management of urethral discharge in South Africa; prior to that it was not widely available for other indications in the public health sector.[10] Despite the short period of time that azithromycin has been used to treat STIs, we already observed a 15% resistance in azithromycin among our isolates; all of these had been obtained from MSM. Moreover, a substantial number (22%) of *N. gonorrhoeae* strains had elevated MICs (0.5-0.75 µg/mL) with an Ala-39→Thr mutation which would previously have been classified 'resistant' before the removal of EUCAST breakpoint and the introduction of the ECOFF. This highlights the importance of including male groups, and especially MSM, in routine drug surveillance. At this stage it is unclear to what extent azithromycin resistance is restricted to core group populations or whether it is extended across the general population in South Africa. National surveillance among symptomatic individuals at sentinel facilities suggest that there is still a low prevalence (<3%) of azithromycin intermediate resistance of *N. gonorrhoeae*, however, core transmission groups are not specifically included.[45] On the other hand, recent data from two clinics in the Kwazulu-Natal province suggest the opposite as they detected azithromycin resistance in 68% of isolates.[46] This study used agar dilution methods for MIC determination rather than E-test. Nevertheless, the three studies provide a clear signal that intensified monitoring of azithromycin resistance is highly warranted as emerging resistance may undermine the effectiveness of syndromic management. Whereas the UK, USA and Australian STI treatment guidelines are abandoning the principle of dual therapy for gonorrhoea, recommending only ceftriaxone, dual therapy of azithromycin and ceftriaxone will remain in effect in settings of syndromic management. Good news in that regard is that we did not detect any resistance for the cephalosporins or spectinomycin.

Genomic analysis of *N. gonorrhoeae* strains obtained in this relatively small group of participants shows a genetically diverse bacterial population. We identified a substantial number of novel NG-STAR and NG-MAST STs that have not been reported from elsewhere in the world. This emphasizes the importance of inclusion of local strain libraries and patients when considering new drugs or treatment regimens. Our study is one of the first studies in Africa to perform WGS of *N. gonorrhoeae* isolates and the first that successfully used culture-independent NG-MAST which

could constitute an important low-cost alternative to WGS in resource-constraint settings. Our study demonstrates that genomic analysis of *N. gonorrhoeae* strains circulating in core transmission groups can be achieved in a low-resource setting and that it may provide important additional insights in the occurrence and basis of antimicrobial resistance.

This study has several limitations, especially the relatively small sample size. For reasons of feasibility we aimed at recruiting high-risk men from core transmission groups, at which we succeeded. Although uptake was high, these groups are difficult to target in the South African public health setting. However, despite the low number of participants, this study provides a very comprehensive overview of the *N. gonorrhoeae* strains circulating in this population with a concerning message suggesting emerging drug resistance. Second, despite direct inoculation on agar plates, microbial culture remained negative in about one-third of participants with a positive molecular test for *N. gonorrhoeae*. This reflects the challenges of obtaining specimens in a clinic and transporting these to the laboratory in low-resource settings. Based on the overlap in NG-MAST STs we have no reason to assume that the uncultivated strains are different from those that were cultivated.

Antimicrobial resistance in *N. gonorrhoeae* with the threat of untreatable gonorrhoea is a global health concern. Our study provides a clear signal that a stronger focus is required on this infection in low-resource settings including the public healthcare sector in South Africa. We confirm the high resistance rates to ciprofloxacin and tetracycline which have been used for treatment of STIs for many years in South Africa. However, the emergence among MSM of azithromycin resistance only a few years after its introduction in syndromic management is highly concerning. There are no data on azithromycin resistance in this population prior to its introduction, however, national surveillance reported a prevalence of less than 3%.[45] Many MSM in our setting have both male and female sex partners facilitating transmission from this relatively contained group to the general population. With an epidemic of MDR gonorrhoea looming it is imperative to strengthen sexual healthcare services in the country. Intensified clinical governance and antimicrobial stewardship, introduction of molecular diagnostics, careful selection of empirical treatment regimens, evaluation of new potential drugs such as zoliflodacin, and investment in an enhanced antimicrobial surveillance structure that includes core transmission groups is essential to pre-empt such development and to avoid an epidemic of untreatable gonorrhoea in sub-Saharan Africa.

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CHAPTER 4

Failure of syndromic management due to drug-resistant *Mycoplasma genitalium* infection in South Africa: a case report

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Abstract

We report a case of management failure of male urethritis syndrome due to macrolide resistant *Mycoplasma genitalium* in South Africa. We detected mutations in 23S rRNA and one of the quinolone resistance-determining regions. This report confirms that drug-resistant *M. genitalium* infection can undermine effectiveness of syndromic management in Africa.

4.1 Introduction

In South Africa, sexually transmitted infections (STIs) are managed syndromically through algorithms based on the patient's presenting symptoms. Male urethritis syndrome (MUS) is a common clinical presentation at primary healthcare facilities in South Africa; it is usually caused by *N. gonorrhoeae, C. trachomatis*, *T. vaginalis* or *M. genitalium* infection [1] *M. genitalium* infection is treated by azithromycin in the syndromic management regimen. However, there have been reports of widespread treatment failure of azithromycin for male urethral infection from across the globe, but not yet from the African continent where syndromic management is used [2]. We present a case of syndromic treatment failure due to drug-resistant urethral *M. genitalium* infection and discuss the implications of this for the management of STIs in resource-constraint settings.

4.2 Case report

A 25-year-old male, HIV-negative, presented at a community health centre (CHC) in Soweto, South Africa, with symptoms suggestive of MUS. He complained of dysuria and penile discharge for the last 14 days. The patient reported one stable female sexual partner that did not have symptoms. He was treated syndromically for MUS with 250 mg ceftriaxone intramuscular injection and 1 g azithromycin as per the current South African STI management guidelines [3]. In addition, a partner notification slip was issued to allow him to notify his sexual partner.

Four weeks later, the patient returned to the same CHC complaining of persistent dysuria and penile discharge. The symptoms remained unchanged following initial syndromic treatment and he was unwilling to disclose and notify his sexual partner. Discharge was confirmed during examination; no other abnormalities were identified. The patient was enrolled in a study addressing the aetiology of persistent symptomatic STIs in men in Johannesburg. After informed consent was obtained, a sample of urethral discharge and first-void urine (FVU) were obtained for further investigation. In line with the South African STI management guidelines, the patient was treated with an increased dosage of 1 g of ceftriaxone combined with a single dose azithromycin 2 g.

The urethral discharge swab was plated on New York City agar for culture of *N. gonorrhoeae*; no growth was observed after 48 hrs. FVU was used for molecular detection of *C. trachomatis*, *N.*

gonorrhoeae, and *T. vaginalis* using the PrestoPlus CT-NG-TV assay (Microbiome Ltd, Houten, the Netherlands)[4]; all reactions were negative. *M. genitalium* infection, however, was detected from the urine sample using a validated in-house *M. genitalium* PCR assay [5]. *M. genitalium* DNA was tested for macrolide drug resistance-associated mutations in the 23S rRNA gene using real-time PCR coupled with melting curve analysis [6]. Melting curve analysis of the DNA showed a peak (63.7°C) differing from the wild type strain (68.3°C). Subsequently, a 283 bp fragment of the 23S rRNA gene was amplified in samples with a temperature peak different from the wild-type strains using primers MG-23S-fw GAAGGTTAAAGAAGGAGGTTAGCAAT and MG-23S-rev CTACCTATTCTCTACATGGTGGTGTTT followed by sequencing [7]. Sequences were aligned against the *M. genitalium* reference strain G-37 23S ribosomal RNA gene (NR_077054.1) using MEGA7 [8]. Mutations were reported using *M. genitalium* numbering. Sequence analysis of the 23S rRNA gene confirmed a mutation at position A2071G, associated with macrolide resistance (**Table 4.1**) [6, 9]. A quinolone resistance-associated mutation was detected by sequencing of quinolone-resistance determining regions of the *parC* and *gyrA* genes [10]. Nucleotides 172–402 of *gyrA* and 164–483 of *parC* were amplified [9], followed by sequencing. Sequences were aligned against the *M. genitalium* reference strains: CP003773 for *gyrA* and *parC for U25549.* The patient was contacted telephonically to discuss his results and was advised to return to the clinic for retreatment. Unfortunately, the patient did not return for further treatment and could no longer be reached telephonically.

Table 4.1 Mutations associated with macrolide and quinolone resistance in *Mycoplasma genitalium* infection in a patient failing management of male urethritis syndrome in South Africa

Drug resistance associated alterations in *Mycoplasma genitalium*		
23S rRNA gene[a]	***parC* gene[a]**	***gyrA* gene**
A2071G	C234T (Pro-62→Ser)[b]	Wild type

[a] Nucleotide positions according to *M. genitalium* numbering according to G37 reference genome (NC_000908).

[b] Numbering of amino acids according to *M. genitalium* gene sequences

4.3 Discussion

We present a case of failure of syndromic management of male urethritis due to macrolide-resistant *M. genitalium* infection in South Africa. In addition, a mutation associated with quinolone resistance was identified that has been reported from various countries across the globe [9]. To our knowledge, a case of male urethritis by multi-drug resistant *M. genitalium* infection has not been reported from the African continent before. In the absence of routine laboratory diagnostics in STI care, antimicrobial resistance data of *M. genitalium* infections in Africa is scarce and limited to small number of studies of reporting asymptomatic *M. genitalium* infections in women [11, 12].

The case that we report highlights a potential limitation of syndromic management in the context of macrolide-resistant *M. genitalium* infection. As per guidelines, our patient was treated twice with azithromycin and ceftriaxone, but without success. Moxifloxacin is not included in the current algorithm nor is it routinely available in public care in South Africa, although its effectiveness in this case is uncertain. Diagnostics are not routinely available in the public healthcare system in South Africa and, if done, usually only focus on the cultivation of *N. gonorrhoeae*. As such, it is highly unlikely that our patient would have received adequate antimicrobial treatment.

The widespread use of azithromycin in the syndromic management of STIs has significantly enhanced the spread of macrolide-resistant *M. genitalium* infections [13]. As debated by others, and confirmed by us through this case report, drug-resistant *M. genitalium* infection has the potential to undermine the effectiveness of syndromic management of STIs [14]. Epidemiological data on the prevalence and distribution of macrolide-resistance in the African context are urgently warranted to inform syndromic management guidelines. Important considerations in that regard are the implementation of aetiological testing that should include *M. genitalium* and potential drug resistance, as well as the establishment of a strengthened second-line algorithm for those that fail initial syndromic management.

4.4 Ethical approval

The study was approved by the Research Ethics committee, Faculty of Health Sciences, University of Pretoria (Ref 253/2017).

4.5 Acknowledgement

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4.6 Declaration of Conflicting Interests

The Authors declare that there is no conflict of interest.

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CHAPTER 5

Molecular epidemiology of *Mycoplasma genitalium* infections in South Africa shows low prevalence of azithromycin resistance and a well-established epidemic

The editorial style of the Journal of Infectious Diseases has been followed for this chapter.

Key messages

- Molecular typing of *Mycoplasma genitalium* strains demonstrates a genetically diverse epidemic without geographic clustering in South Africa.
- Resistance to azithromycin is still uncommon in *Mycoplasma genitalium* infections in individuals attending the public healthcare sector.
- Azithromycin resistance, although uncommon, in *Mycoplasma genitalium* can undermine the effectiveness of syndromic management in these settings.
- Introduction of diagnostics and enhanced surveillance is warranted for early detection of emerging drug resistance.

Abstract

Objectives. Macrolide resistance in *Mycoplasma genitalium* is emerging globally. There is paucity of data from sub-Saharan Africa where syndromic management is used to treat sexually transmitted infections (STIs). We conducted a molecular epidemiological study to determine the prevalence of azithromycin resistance and epidemic diversity of *M. genitalium* infections in South Africa.

Methods. We analysed 90 *M. genitalium*-positive specimens that had been collected from men and women (50% symptomatic) from geographically diverse communities across the northern part of South Africa between 2015 and 2019. Melting curve analysis followed by targeted sequencing of the 23S rRNA gene was performed to detect mutations associated with azithromycin resistance. Molecular typing was done through single nucleotide polymorphism (SNP) analysis of the MG191 gene and short tandem repeats (STR) assessment of the MG309 gene. An overview of all published *M. genitalium* sequence types was generated and novel sequence types identified in this study were allocated numbers accordingly.

Results. Azithromycin resistance was detected in 1/90 *M. genitalium*-positive specimens (1.1%; 95% confidence interval 0-3.3%) as conferred by A2071G mutation; this strain also harboured a C234T mutation in the *parC* gene with wild type *gyrA* gene. SNP typing and STR assessment was successful in 38/90 specimens (42%) and showed a genetically diverse epidemic, without geographic clustering, with eight novel sequence types identified.

Conclusion. This is the first study that determined resistance in *M. genitalium* infection since the introduction of azithromycin in the syndromic management regimen for STIs in South Africa in 2015. Despite a well-established epidemic, azithromycin-resistant *M. genitalium* infection is still uncommon in the public healthcare sector. However, it has the potential to undermine the effectiveness of syndromic management. Introduction of molecular diagnostics and continuous surveillance are warranted for early detection emergence of resistance.

Keywords

Mycoplasma, molecular epidemiology, azithromycin, molecular typing, Africa

5.1 Introduction

Mycoplasma genitalium is a sexually transmitted, fastidious bacterium associated with genital and urinary tract infection in men and women [1]. Infection may present as discharge or dysuria but can also occur without symptoms. Untreated *M. genitalium* infection is associated with various reproductive tract complications [1]. In South Africa, the prevalence of *M. genitalium* infection in the sentinel aetiological surveillance was reported at 8.6% among women with vaginal discharge and 8.1% in men with urethral discharge [2,3]; research studies reported asymptomatic *M. genitalium* infection among 7.4% of women and 5.3% of men [4,5]. In South Africa, sexually transmitted infections (STIs) including *M. genitalium* are treated syndromically, i.e. with an empirical combination of broad-spectrum antibiotics based on the presenting symptoms [4]. Azithromycin and ceftriaxone, often combined with metronidazole, are the current syndromic regimen of choice for urethral and vaginal discharge.

Treatment of *M. genitalium* infection has become increasingly challenging worldwide due to emergence of resistance to azithromycin, the first-line therapeutic choice [1]. Resistance to azithromycin and other macrolides is mainly mediated by mutations in the V-domain of the 23S ribosomal RNA (rRNA) gene; these mutations can be detected reliably using various molecular methods [6,7]. Two brief reports from South Africa describing a total of six asymptomatic women with azithromycin-resistant *M. genitalium* infection confirms that such infections might occur [6,7]. However, in the absence of routine diagnostics, the prevalence of azithromycin resistance in *M. genitalium* infections in sub-Saharan Africa is unknown. In order to optimally support clinical management of patients, guide the design of syndromic management algorithms, and to inform public health programmes, measuring the population-level prevalence of azithromycin resistance is essential.

Understanding of the spread of azithromycin-resistant *M. genitalium* infection could be enhanced by molecular typing of strains to establish epidemic diversity. A well-established method for molecular typing of *M. genitalium* is the combination of single nucleotide polymorphism (SNP) analysis of the MG191 gene and a short tandem repeat (STR) assessment of the MG309 gene [8]. This technique has been used by various researchers to report sequence types (STs) [8].

This study measured the prevalence of azithromycin resistance in *M. genitalium* among infected patients from a wide geographic area of northern South Africa. Molecular typing of *M. genitalium* was performed and a review of globally reported STs was compiled with the aim of gaining insight into the *M. genitalium* epidemic and associated azithromycin resistance.

5.2 Methods

5.2.1 Description of study cohorts that provided specimens for this analysis

We analysed 90 stored specimens from individuals with *M. genitalium* infection recruited into four studies conducted in Tshwane and Johannesburg Health Districts, Gauteng Province, and in the Mopani District, Limpopo Province, South Africa; these studies are summarized in **Table 5.1** [9-13]. The prevalence of *M. genitalium* in these study cohorts ranged from 7.0 to 15%. Fourteen strains (16%) were obtained from men and 76 (84%) from women; infections were symptomatic in 45/90 (50%) of cases. Gauteng Province facilities (Tshwane and Johannesburg Health Districts) were ~90-120 km apart. Mopani District is ~350-450 km north of Tshwane and Johannesburg, and facilities there were up to 100 km apart from each other. In all studies *M. genitalium* detection was performed at the Department of Medical Microbiology, University of Pretoria, using a validated real-time PCR targeting the *MgPa* gene on the LightCycler assay (Roche Molecular Diagnostics, Germany) after DNA extraction using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Switzerland) [14]. Stored positive DNA specimens from these studies were used in this evaluation.

5.2.2 Detection of macrolide resistance-associated mutations

We used the modified real-time PCR assay as described by Xiao et al. for detection of macrolide resistance-associated mutations [15]. This assay uses real-time PCR based on fluorescence resonance energy transfer (FRET) technology targeting the relevant section of the 23S rRNA gene of *M. genitalium* coupled with melting curve analysis for detection of point mutations. Amplification curves were analysed using the Abs quant/2nd Derivative Max and Melting temperatures (T_m) values were determined by using the manual T_m function. Thereafter, a 283 bp fragment of the 23S rRNA gene was amplified in samples with a temperature peak different from the wild-type strains using primers MG-23S-fw GAAGGTTAAAGAAGGAGGTTAGCAAT and

MG-23S-rv CTACCTATTCTCTACATGGTGGTGTTT followed by sequencing [16]. Quinolone resistance-associated mutations were detected by sequencing of quinolone-resistance determining regions (QRDR) of *gyrA* and *parC* genes. Nucleotides 172–402 of *gyrA* and 164–483 of *parC* were amplified, followed by sequencing [17]. To confirm reproducibility of the melting curve analysis results we performed the multiplex ResistancePlus^{TM} qPCR assay (SpeeDx Pty Ltd, Australia) on a random subset of 30 specimens at the Department of Medical Microbiology at the Maastricht University Medical Centre.

5.2.3 Molecular epidemiology of *Mycoplasma genitalium*

We used SNP analysis of the MG191 (*mgpB*) gene in the MgPa operon combined with STR assessment of the putative lipoprotein MG309 gene for molecular typing of *M. genitalium* at the Department of Medical Microbiology at the Maastricht University Medical Centre [8].

Following SNP analysis, we generated a dendrogram to visualise the relationship between *M. genitalium* specimens using the unweighted pair-group method with arithmetic mean. A ST was assigned to each *M. genitalium* strain using the numbering system developed by Cazanave *et al.* [8]. This system includes STs number 1-56 from a study conducted by Hjort *et al.* and STs 57-60 by Ma *et al*. [18,19]. We allocated number 64-80 to STs described by Musatovova, 81-88 for STs published by Cazanave, and 89-98 for those reported by Pond *et al*. [8,20,21]. As such, the numbering of new STs identified in our study starts from 99 onwards. As for STR analysis, the number of tandem repeats in the MG309 gene was counted and reported. *Mycoplasma genitalium* strains with macrolide resistance-associated mutations were visualised in the dendrogram to identify potential clustering and assess genetic relatedness.

5.2.4 Statistical analysis

Descriptive statistics are provided as number with proportion, median with range and mean with standard deviation. We did not perform any comparative statistics.

5.2.5 Ethical approval

The collection of cohorts included in this study was approved through ethical approval by the Research Ethics Committee at the Faculty of Health Sciences of the University of Pretoria (Reference numbers 253/2017, 498/2016, 401/2015), the Human Research Ethics Committee of the University of the Witwatersrand, Johannesburg, South Africa (Ref: M150352).

Table 5.1 Overview of study cohorts from which *Mycoplasma genitalium* specimens (n=90) are included in this analysis

Year	Geographic location	Setting	Sample size	Patient population	*M. genitalium* prevalence	Symptomatic infection	Ref
2015-2019	Johannesburg Health District	Male sexual health services at three PHC facilities	129	Men with urethral or anal discharge	7.0% (n=9)	9/9 (100%)	9,10
2016	Mopani District	Mobile clinic service in deep-rural areas	251	Women attending for any type of health services	8.4% (n=21)	11/21 (52%)	11
2016-2017	Tshwane Health District	Three PHC facilities in urban township	294	HIV-infected pregnant women attending ANC	15% (n=44)	9/44 (20%)	12
2017-2018	Mopani District	Six PHC facilities in rural areas across the district	177	Men and women with STI symptoms mobilised for care	9.0% (n=16)*	16/16 (100%)	13

*Eleven women and five men.

Note. PHC, primary health care; ANC, antenatal care; STI, sexually transmitted infection

5.3 Results

5.3.1 Prevalence of macrolide resistance

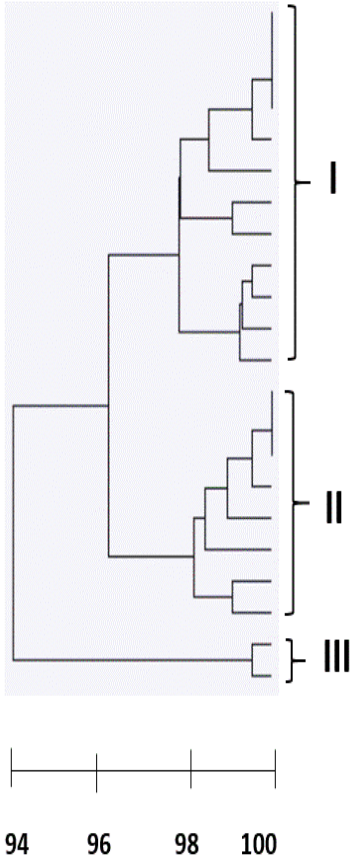
Ninety *M. genitalium*-positive specimens were included in the analysis. In melting curve analysis, 89/90 (99%) of *M. genitalium* specimens showed a peak melting temperature in similar range to the wild-type strain suggesting the absence of macrolide resistance-associated mutations. Only one specimen (1.1%; 95% confidence interval 0-3.3%) showed a melting peak (63.7°C) different from that of the wild type strains (68.2°C ±0.5). Sequence analysis of the 23S rRNA gene confirmed an A2071G mutation associated with macrolide resistance. Sequencing of the quinolone resistance-determining regions revealed a wild type *gyrA* gene and a C234T mutation in the *parC* gene resulting in a proline to serine substitution. Macrolide resistance results were concordant between melting cure analysis and the multiplex ResistancePlus^{TM} real-time PCR assay (qPCR).

5.3.2 Molecular epidemiology of *Mycoplasma genitalium*

Genotyping was successful in 38/90 (42%) specimens; successfully genotyped specimens had cycle threshold values <38 in the initial diagnostic *MgPa*-targeted qPCR assay. Single nucleotide polymorphism analysis of MG191 was successful in 22/90 specimens (24%) and revealed 17 different STs comprising three clusters (**Figure 5.1**). Strain clustering by geographic location was not observed; ST-2 and ST-7 were observed in specimens from all three geographic locations. Eight novel STs were identified, numbered 99 to 106, and deposited sequences into GenBank under the accession numbers MN543061, MN543062, MN543063, MN543064, MN543065, MN543066, MN543067 and MN543068. All *M. genitalium* STs previously assigned in the literature, and newly identified in this study, can be found in Table 5.2 S1 (Online supplementary data A).

MG309STR analysis was successful in 35/90 (39%) specimens of which 19 (54%) had MG191 STs previously allocated. The number of STRs observed varied between 8 and 19. As MG309 contains two different repeat units, AGT and AAT, we examined the distribution patterns of these units between different specimens. Specimens with 9 or 14 STRs showed three different AGT/AAT distribution patterns, whereas two different patterns were observed for those with 16 STRs. The AGT/AAT distribution was identical in specimens with 8, 11 or 15 STRs.

Combining the number of SNPs and STR resulted in identification of unique STs. The azithromycin resistant *M. genitalium* strain was a novel ST (# 101) and had 11 STRs.



Study site	MG191 ST	MG309 STR	Gender	HIV status
Pretoria	7	11.1	Female	Positive
Mopani District PHC	7	11.1	Male	Positive
Johannesburg	7	15.1	Male*	Positive
Johannesburg	7	11.2	Male	Unknown
Johannesburg	89	12.1	Male	Negative
Mopani District PHC	6	17.1	Male	Positive
Mopani District MHC	3	14.1	Female	Negative
Johannesburg	99	8.1	Male*	Positive
Pretoria	65	19.1	Female	Positive
Johannesburg	4	14.2	Male*	Negative
Pretoria	100	9.1	Female	Positive
Johannesburg	101	11.2	Male	Negative
Pretoria	2	15.1	Female	Positive
Mopani District PHC	2	14.3	Male	Positive
Johannesburg	2	-	Male*	Negative
Pretoria	102	-	Female	Positive
Pretoria	64	13.1	Female	Positive
Pretoria	103	-	Female	Positive
Pretoria	104	8.1	Female	Positive
Mopani District MHC	105	10.1	Female	Negative
Pretoria	51	16	Female	Positive
Mopani District MHC	106	9.2	Female	Negative
Pretoria	-	8.1	Female	Positive
Pretoria	-	9.3	Female	Positive
Pretoria	-	9.3	Female	Positive
Pretoria	-	9.3	Female	Positive
Mopani District MHC	-	10.2	Female	Negative
Pretoria	-	11.2	Female	Positive
Pretoria	-	11.2	Female	Positive
Mopani District MHC	-	12.2	Female	Positive
Pretoria	-	13.2	Female	Positive
Mopani District PHC	-	14.2	Female	Positive
Mopani District PHC	-	14.4	Female	Negative
Mopani District PHC	-	15.2	Female	Positive
Pretoria	-	16.2	Female	Positive
Pretoria	-	16.3	Female	Positive
Mopani District MHC	-	16.4	Female	Negative
Mopani District MHC	-	16.5	Female	Negative
Mopani District MHC	-	14.4		
Mopani District MHC	-	14.2		

Figure 5.1 Dendrogram of *Mycoplasma genitalium* specimens obtained from different areas and participant populations in South Africa. Underlined sequence type numbers (99- 106) indicate novel sequence types.

Abbreviations. ST, sequence type; STR, short tandem repeat; HIV, human immunodeficiency virus; PHC, primary healthcare clinic; MHC, mobile healthcare clinic.

*Men reporting having sex with other men.

5.4 Discussion

This study is among the first to address the molecular epidemiology of *M. genitalium* infection in sub-Saharan Africa. We demonstrate that, although azithromycin resistance is still uncommon, there is a well-established epidemic of *M. genitalium* infections in the northern part of South Africa. This is based on the wide genetic diversity of *M. genitalium* isolates identified from different geographic areas and populations and the detection of eight novel strains.

Though we observed azithromycin resistance in less than 5% of *M. genitalium* infections, its presence among specimens from populations in the northern part of South Africa is concerning. This contrasts with two prior studies, including one from the national STI surveillance in Gauteng province that includes Johannesburg and Tshwane Health Districts where no azithromycin resistance was identified [4,22]. However, unlike those two studies, the *M. genitalium* strains analysed in this study were collected after the introduction of azithromycin in the STI syndromic management regimen in South Africa in 2015; exposure to this drug is considered an important driver of *M. genitalium* azithromycin resistance emergence worldwide [23]. The low rate of azithromycin resistance among our specimens may be a result of the relatively short period between the introduction of azithromycin as part of syndromic management guidelines and when specimens were collected. Consequently, continuous surveillance is warranted to detect potential emergence of resistance at this early stage, as the emergence of resistance could undermine syndromic management for STIs in our region [24]. However, at this stage azithromycin can still be considered an appropriate antimicrobial to cover *M. genitalium* infection in the syndromic regimen prescribed to individuals presenting with dysuria or discharge in South Africa [10].

This study is among the first molecular epidemiological studies of *M. genitalium* strains using an established dual method of SNP and STR analysis. To achieve this, ST numbering database initiated by Cazanave and colleagues to was expanded to create a comprehensive, global ST database [8]. A substantial proportion of the *M. genitalium* specimens tested in our study were allocated novel ST numbers and had different STR numbers than those reported from previous studies in Europe, the USA and Tunisia [8,18-21]. We identified three major clusters with strains from multiple geographic regions allocated to each cluster. This confirms the widespread nature of individual STs, and the presence of a diverse epidemic rather than clonal spread of

specific strains. The azithromycin-resistant strain, although assigned a novel ST, belonged to a cluster that occurs throughout the region suggesting that resistance could emerge easily following prolonged exposure to azithromycin.

This study has several limitations. First, specimens from several study cohorts were combined for this analysis instead of performing well-designed surveillance. We do think, however, that the diverse cohorts included in this study provide a good representation of the situation in the northern part of South Africa. Second, we only managed to successfully perform genotyping for a proportion of the specimens due to low microbial load and degradation of DNA that had occurred in some specimens. Naturally occurring low load infections are a known limitation when performing molecular epidemiological analysis of *M. genitalium*. However, there is no indication that our interpretation (i.e. a well-established genetically diverse epidemic with multiple clusters) would have been any different should typing of all specimens have been successful.

In conclusion, this study confirms the presence of a well-established epidemic of *M. genitalium* infection in South Africa. Though azithromycin resistance is still uncommon, its detection after the introduction of azithromycin for syndromic management in 2015 may portend its increased emergence over time. Surveillance must be performed for the early detection of emergence of resistance and prolonged use of azithromycin as part of syndromic management of STIs.

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CHAPTER 6

High rates of antimicrobial resistance in *Neisseria gonorrhoeae* and *Mycoplasma genitalium* infections in the private healthcare sector in South Africa

The editorial style of the Sexually Transmitted Infections has been followed in this chapter.

Abstract

Background: *Neisseria gonorrhoeae* and *Mycoplasma genitalium* are important pathogens linked to male urethritis syndrome and vaginal discharge syndrome. In South Africa, sexually transmitted infections (STIs) are treated using the syndromic management approach in the public healthcare sector, accessed by most of the population. In the private sector, there is access to laboratory diagnostics, but syndromic management is used as well. Globally reports have emerged of antimicrobial resistance (AMR) in *N. gonorrhoeae* and *M. genitalium* infections. The aim of this study was to determine the rates of AMR in *N. gonorrhoeae* and *M. genitalium* infections in the South African private healthcare sector.

Methods: *N. gonorrhoeae* and *M. genitalium* isolates were collected from a private diagnostic reference laboratory from August 2018 to June 2019 in this cross-sectional study. *N. gonorrhoeae* antimicrobial susceptibility testing was performed as per EUCAST guidelines, followed by NG-MAST to determine genetic relatedness of the isolates. *M. genitalium* macrolide resistance was determined using a real-time time PCR assay coupled with melting curve analysis and mutations confirmed by sequencing the 23S rRNA gene. Fluoroquinolone resistance-associated mutations were detected by sequencing of quinolone-resistance determining regions (QRDR) of *gyrA* and *parC* genes in macrolide resistant isolates.

Results: Twenty-one *N. gonorrhoeae* and twenty-six *M. genitalium* positive specimens were included in this analysis. Most of *N. gonorrhoeae* isolates were resistant to tetracycline (90%), penicillin (86%) and ciprofloxacin (62%). All strains were susceptible to azithromycin, cephalosporins and spectinomycin. *N. gonorrhoeae* isolates were genetically diverse as

identified using NG-MAST. Macrolide resistance associated mutations in the 23S rRNA were detected in 5/26 (19%) *M. genitalium* strains; three A2071G mutations and two A2072G mutations. Two strains harboured alterations in the GryA of QRDR at position 132 (Gln→Arg) and one strain had a Ser83→Ile substitution in the ParC associated with fluoroquinolone resistance. Dual-class drug resistance to macrolides and fluoroquinolones was detected in two strains.

Conclusions: AMR in *N. gonorrhoeae* and macrolide resistance has emerged in the private healthcare sector. Substantial differences in rates of gonococcal resistance to tetracycline and penicillin were noted compared to reports from national sentinel surveillance. It is imperative to include the private healthcare sector facilities in future national sentinel surveillance for early detection of emerging AMR.

Keywords: *Neisseria gonorrhoeae*, antimicrobial resistance, *Mycoplasma genitalium,* sexually transmitted infections, macrolide resistance, fluoroquinolone resistance

6.1 Introduction

Sexually transmitted infections (STIs) in South Africa are treated syndromically based on the patient's presenting symptoms and using a combination of empirical antibiotics.[1] Syndromic management has several well-documented limitations such as poor specificity leading to inappropriate use of antibiotics (overtreatment) with the risk of antimicrobial resistance development, limited surveillance for detection of antimicrobial resistant strains, inability to detect asymptomatic infections and lack of antimicrobial susceptibility testing.[1 2] *Neisseria gonorrhoeae* and *M. genitalium* are important STI pathogens associated with male urethritis syndrome and vaginal discharge syndrome. *Neisseria gonorrhoeae* infection is treated using a dual therapy of ceftriaxone and azithromycin in the syndromic management regimen while *M. genitalium* infection is covered by azithromycin.[3] However, globally reports have emerged of antimicrobial resistance (AMR) in *N. gonorrhoeae* and *M. genitalium;* presenting a significant public health threat.[4 5] Development of AMR in *N. gonorrhoeae* and *M. genitalium* is directly linked to (over-) consumption of antibiotics or through the bystander effect, defined as antibiotic selection pressure due to treatment of unrelated infection. [4 6]

Data from national STI surveillance do not show macrolide and fluoroquinolone resistance in *M. genitalium* resistance in South Africa.[7 8] However, macrolide resistant *M. genitalium* infections have been reported in small series of asymptomatic women in South Africa.[9 10] With regard to AMR *N. gonorrhoeae,* the national sentinel surveillance reported azithromycin resistance prevalence of less than 5% from 2008-2017.[11] However, high levels of azithromycin resistance have been reported in recent studies; a study in the KwaZulu-Natal province reported azithromycin resistance prevalence of 68%[12] and another one in Gauteng reported a prevalence of 15% (Maduna *et al* submitted for publication). In addition, the first two cases of cefixime-resistant *N. gonorrhoeae* infection in Africa were reported in the private healthcare sector in South Africa. [13 14] South Africa has one of the widest disparities between the public and private health sectors in the world.[15] Only 15% to 17% of the population have full access to private healthcare largely through their own private health insurance, while up to 82% of the population is dependent on state-provided free-of-charge public healthcare.[15 16] In the private healthcare sector; there are more doctors in relation to patient population in private practice than in the public sector even in the rural areas. [16 17] In addition, patients in the private sector are from a higher socio-economic status and have higher utilisation rates than in public health sector for

acute illness.[18] Furthermore, patients from the private healthcare sector have high consultation rates and use self-prescribed antimicrobials than in public healthcare sector. Studies have shown general practitioners (GPs) prescribing behaviour is dependent on whether a patient is insured or uninsured.[17] We conducted a pilot study to determine the rates of AMR of *N. gonorrhoeae* and *M. genitalium* infections in the private sector.

6.2 Methods

In this cross-sectional study, *N. gonorrhoeae* isolates and positive *M. genitalium* DNA samples with linked anonymous clinical information were collected from a private diagnostic reference laboratory from August 2018 to June 2019 in Pretoria, South Africa. This laboratory receives specimens from all provinces in South Africa for pathology services. *N. gonorrhoeae* was cultured on New York City (NYC) agar (Thermo Scientific, USA) from the urine, urethral and endocervical swabs (Copan, Italy) as submitted by the private practitioner. This was followed by identification using Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALD-TOF) (bioMérieux, France). Cultured *N. gonorrhoeae* on NYC agar was transported in an AnaeroPack™ (Thermo Scientific, Lenexa, USA) to the Department of Medical Microbiology at the University of Pretoria for further processing. *M. genitalium* was identified from urine, urethral and endocervical swabs using the Anyplex™ II STI-7 (Seegene, Seoul, Korea) real-time PCR assay and the DNA samples were stored at -20°C freezer. *M. genitalium* DNA was transported in a cooled shipping box to the Department of Medical Microbiology at the University of Pretoria for further processing.

At the University of Pretoria, *N. gonorrhoeae* was confirmed by Gram stain, rapid oxidase test and API® NH (bioMérieux, France). This was followed by antimicrobial susceptibility testing to tetracycline, ciprofloxacin, penicillin G, azithromycin, ceftriaxone, cefixime and spectinomycin. Minimum inhibitory concentration (MICs) were determined using E-test (bioMérieux, France) on the GC-agar base enriched with 1% IsoVitaleX (Oxoid Ltd, UK). MICs were interpreted according to European Committee of Antimicrobial Susceptibility Testing breakpoints (EUCAST), except for azithromycin where epidemiological cut-off (ECOFF = 1 mg/L) values were used because no resistance breakpoints exist (http://www.eucast.org). *N. gonorrhoeae* ATCC 49266 strain was used as quality control strain. Molecular epidemiology of gonococcal isolates was determined using the *N. gonorrhoeae*

multiantigen sequence typing (NG-MAST) as previously described.[19] Briefly, PCR amplification of the *porB* and *tbpB* genes was performed in 50 µL reaction using a SimpliAmp™ Thermal Cycler (ThermoFisher Scientific, USA). Each PCR reaction contained 50 pmol of each primer, 2.5 U HotStar polymerase (Qiagen), 1 X buffer (Qiagen), 0.2 mmol/L dNTP (Applied Biosystems), 2 µL DNA lysate and nuclease free water to a volume of 50 µL. [19] The PCR cycle protocol involved an initial denaturation of 4 min at 95°C, followed by 25 cycles of 30 s at 95°C, 30 s at 58°C, and 1 min at 72°C, followed by a final extension of 10 min at 72°C and cooling to 4°C. [19] The edited and trimmed *porB* and *tbpB* sequences were submitted to the NG-MAST database (http://www.ng-mast.net) for assignment of sequence types. Trimmed *porB* and *tbpB* sequences were concatenated and a maximum likelihood phylogenetic tree was constructed using MEGA v 7.0.[20]

Mycoplasma genitalium was confirmed using a validated real-time PCR assay targeting the *MgPa* adhesin gene.[21] Macrolide drug resistance associated mutations in the 23S rRNA gene were identified using real-time PCR assay coupled melting curve analysis for detection of point mutations.[22] Subsequently, a 283 bp fragment of the 23S rRNA gene was amplified in samples with a temperature peak different from the wild-type strains using primers MG-23S-fw GAAGGTTAAAGAAGGAGGTTAGCAAT and MG-23S-rv CTACCTATTCTCTACATGGTGGTGTTT followed by sequencing [5]. Sequences were aligned against the *M. genitalium* reference strain G-37 23S ribosomal RNA gene (NR_077054.1) using MEGA7[20]. Mutations were reported using *M. genitalium* numbering. Quinolone resistance-associated mutations were detected by sequencing of quinolone-resistance determining regions (QRDR) of *gyrA* and *parC* genes as previously described. Briefly, nucleotides 172–402 of *gyrA* and 164–483 of *parC* were amplified, followed by sequencing[5]. Sequences were aligned against the *M. genitalium* reference strains: CP003773 for *gyrA* and *parC* for U25549. Ethical approval was obtained from the Research Ethics committee at the Faculty of Health Sciences, University of Pretoria (Reference numbers: 253/2017, 207/2018 and 171/2019).

6.3 Results

Twenty-one *N. gonorrhoeae* isolates were collected; the majority of the isolates (90%) were from males **(Table 6.1**). *N. gonorrhoeae* was confirmed in 21/21 (100%) of all specimens

received. **Table 6.2** shows the antimicrobial susceptibility patterns for the antimicrobials tested; gonococcal isolates showed high rates of resistance to tetracycline (19/21;90%), penicillin (18/21;86%) and ciprofloxacin (13/21;62%). All isolates were susceptible to azithromycin, cephalosporins and spectinomycin. The NG-MAST classified 18 isolates into 12 different sequence types (STs); ten (10/12;83%) of these STs have not been previously described. Amplification of *porB* gene in one and *tbpB* gene in two isolates failed. The common STs were ST3609 (n=3 isolates), ST6216 (n=2), novel ST containing *porB* allele 1320 and *tbpB* allele 623 (n=2), and a novel ST containing *porB* allele 11132 and *tbpB* allele 136. Overall, 11 different *porB* alleles and 12 *tbpB* alleles were identified. **Figure 6.1** shows a maximum likelihood tree of concatenated *porB* and *tbpB* genes showing diversity between the NG-MAST STs.

Table 6.1 Demographic characteristics of the study population (n=48)

Characteristics	*Neisseria gonorrhoeae* n=21	*Mycoplasma genitalium* n=27
Median age (range)	32 (18-49)	26 (20-49)
Sex		
Male	19 (90)	19 (70)
Female	2 (10)	8 (30)
Location (province)		
Gauteng	17 (81)	20 (74)
Western Cape	0 (0)	5 (18)
Eastern Cape	3 (14)	1 (4)
North West	1 (5)	1 (4)
Specimen type		
Urine	0 (0)	21 (78)
Urethral swab	19 (90)	2 (7)
Vaginal swab	2 (10)	4 (15)

Table 6.2 Antimicrobial susceptibility profiles and minimum inhibitory concentrations of *Neisseria gonorrhoeae* isolates collected from private practice in South Africa (n=21)

Drug	Susceptible n (%)	Resistant n (%)	Median MIC* value (µg/mL)	MIC* range (µg/mL)
Tetracycline	2 (10)	19 (90)	16	0.032-32
Penicillin	3 (14)	18 (86)	2	0.016-12
Ciprofloxacin	8 (38)	13 (62)	0.75	0.002-12
Azithromycin*	21 (100)	0 (0)	0.094	0.023-0.75
Spectinomycin	21 (100)	0 (0)	6	3-8
Cefixime	21 (100)	0 (0)	0.016	0.016
Ceftriaxone	21 (100)	0 (0)	0.002	0.002-0.004

***Note.** MIC, minimum inhibitory concentration.

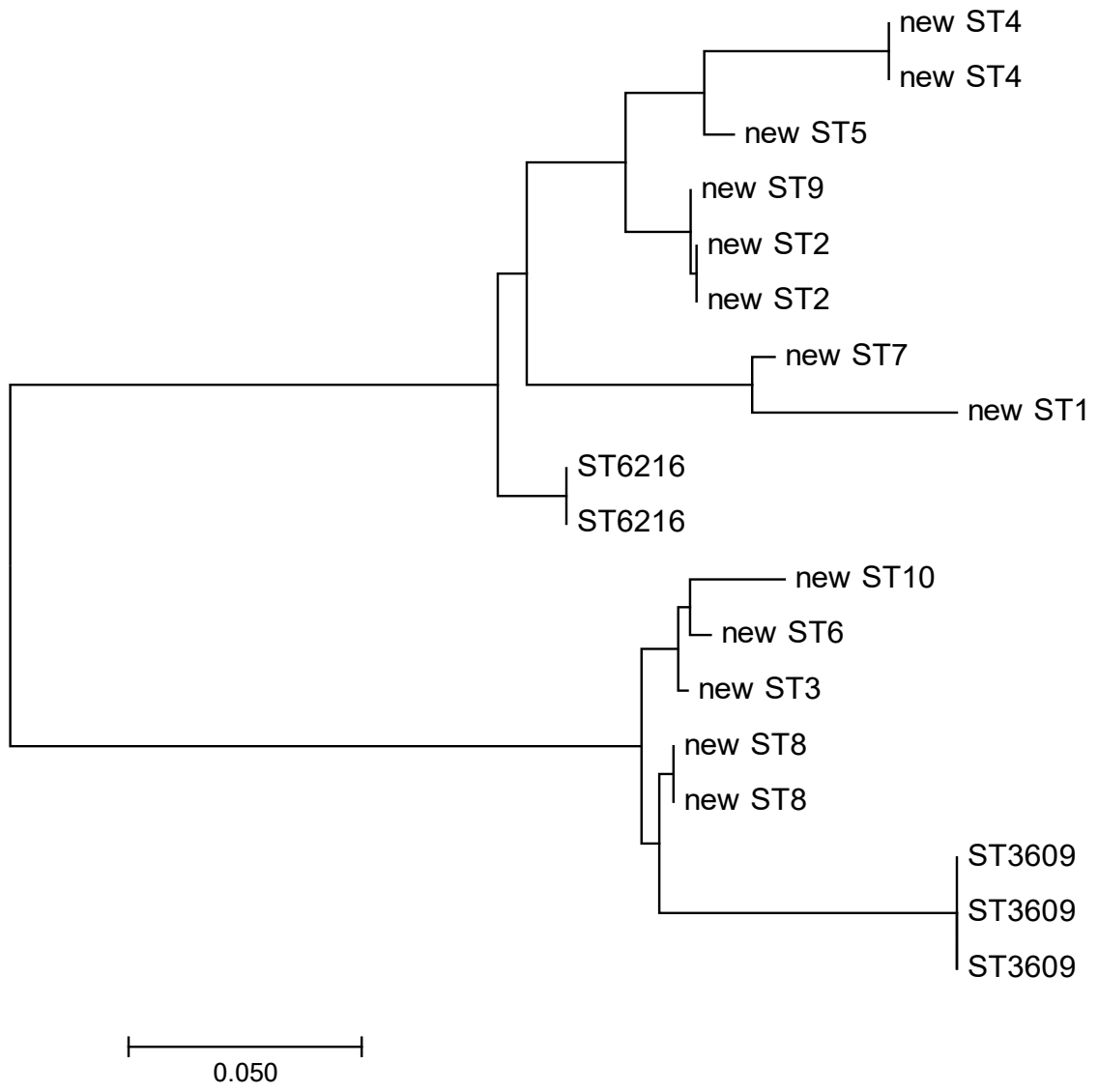

Figure 6.1 Maximum likelihood tree of concatenated sequences of *porB* and *tbpB* genes showing/depicting the diversity of *Neisseria gonorrhoeae* isolates from private healthcare sector in South Africa. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site

Twenty-seven *M. genitalium* isolates were collected, most of the isolates (70%) were from the males **(Table 6.1).** *M. genitalium* was confirmed in 27/27 (100%) of DNA samples received. Melting curve analysis of DNA showed that five strains had a different melting temperature compared to the wild type strain. Sequence analysis confirmed macrolide resistance associated mutations in the 23S rRNA; an A-to-G transition at nucleotide position 2071 was detected in three strains and at position 2072 in two *(M. genitalium* numbering) (**Table 6.3**). Furthermore, various amino acid alterations were observed in the ParC and GyrA of QRDR; two strains had a Gln-132→Arg substitution in the GyrA and the other one had a silent mutation (A318G). In addition, one strain had a Ser83→Ile substitution in the ParC that has been associated with moxifloxacin and sitafloxacin treatment failures.[23] One isolate had novel Met-55→Leu substitution in the ParC of unknown clinical significance. Multidrug resistant *M. genitalium* defined as *M. genitalium* strains with co-existent macrolide and quinolone resistance associated mutations was identified in two patients (**Table 6.3**). [24]

Table 6.3 Macrolide resistance associated mutations and fluoroquinolone resistance associated amino acid changes in GyrA and ParC in *Mycoplasma genitalium* from private healthcare sector in South Africa.

Patient ID	Location of isolate collection	Gender	Specimen type	23SrRNA* mutation	Amino acid change*	
					GyrA	ParC
S1	Western Cape	M	Urine	A2071G	Wild Type	Wild Type
S2	Western Cape	M	Urine	A2071G	Gln-132→Arg	Met-55→Leu
S3	Gauteng	M	Urine	A2072G	Wild type	Wild type
S4	Gauteng	M	Urethral swab	A2072G	Silent mutation[a]	Wild type
S5	Gauteng	M	Urethral swab	A2071G	Gln-132→Arg	Ser83→Ile

[*] Nucleotide positions or amino acid changes numbering according to *M. genitalium* G37 genome (NC_000908.2).

[a] Silent mutation (A318G)

6.4 Discussion

This is first study in South Africa to report on antimicrobial resistant *M. genitalium* infections in patients accessing the private healthcare sector. Here, we demonstrate high rates of AMR in *N. gonorrhoeae* isolates for some of the commonly used antibiotics and dual-class resistance in *M. genitalium* strains, which is much higher than reported in the public healthcare sector. [7 8]

High rates of resistance to tetracycline and ciprofloxacin were detected among gonococcal isolates; these antimicrobials were extensively used in the syndromic management in South Africa in the past decade.[11] These findings are consistent with national surveillance data and a recent study in Kwazulu-Natal province showing high rates of resistance to these antimicrobials.[11 12] In addition, unexpectedly high rates of resistance to penicillin (89%) were observed in gonococcal isolates in this study compared to data from the national sentinel surveillance where a penicillin resistance prevalence of 51% is reported.[11] These findings might be explained by a possibly higher consumption of amoxicillin and amoxicillin/clavulanic acid in the private sector for other unrelated infections such as respiratory tract infections. We did not observe azithromycin resistance in these selected group of isolates from the private healthcare sector. These findings are unexpected as there are concerns about emerging azithromycin resistance in the public healthcare sector.[12] This might be explained by that, in the private healthcare sector there is better targeted treatment for patients due to access to laboratory diagnostics and thereby reducing the antibiotic selection pressure. A positive finding is that all gonococcal isolates were fully susceptible to the cephalosporins and spectinomycin. Molecular epidemiological analysis of the gonococcal isolates showed genetic diversity with a substantial number of novel NG-MAST STs which have not been described elsewhere.

Unexpectedly high rates of macrolide resistance to *M. genitalium* were observed, this is in stark contrast to previous reports from the public healthcare sector where no azithromycin resistance was reported.[7 8] However, azithromycin-resistant *M. genitalium* has been documented in South Africa in a few cases of asymptomatic infections in women.[9 10] The observed high rate of azithromycin resistance in the private healthcare sector is most likely due to different prescribing behaviour; azithromycin is commonly used for treatment of respiratory infections in the private healthcare sector but not in the public healthcare sector.[17] Of great concern is that strains with co-existent macrolide and quinolone resistance associated mutations were detected.

This makes *M. genitalium* infections increasingly difficult to treat given that moxifloxacin is the second-line treatment for macrolide-resistant *M. genitalium* infections and other alternate antimicrobials are not routinely available in healthcare in South Africa. Therefore, this study highlights the important limitations of the syndromic management in the context of resistant *M. genitalium* infections and the urgent need for strengthened laboratory diagnostics coupled with detection of resistance markers.

This study has several limitations. Firstly, the relatively small sample size and geographic spread may not be fully representative of the population in the private healthcare sector; however, it constitutes an important finding in antimicrobial resistant *N. gonorrhoeae* and *M. genitalium* infections in the private healthcare sector. Secondly, in clinical practice, there is selection of patients for laboratory diagnostics as these are expensive and general practitioners generally provide empirical treatment first. Lastly, the therapeutic outcomes of the patients with dual-class resistance in *M. genitalium* associated with treatment failures were not available.

In conclusion, AMR in *N. gonorrhoeae* and macrolide resistance is emerging in the private healthcare sector. Substantial differences in rates of gonococcal resistance to tetracycline and penicillin were noted compared to reports from national sentinel surveillance. These findings highlight the need to scale-up access to and use of diagnostics in the private sector. It is imperative to include the private healthcare sector facilities in future national sentinel surveillance for early detection of emerging AMR.

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CHAPTER 7

Impact of specimen storage temperature and time on the implementation of GeneXpert® testing for sexually transmitted infections in resource-constraint settings

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Abstract

Implementation of routine laboratory diagnostics is imperative to address the high burden of sexually transmitted infections (STI) in Sub-Saharan Africa. We demonstrate that logistical challenges of specimen storage, temperature and transport time are unlikely to impact on performance of routine STI diagnostics using the GeneXpert® platform implemented in these settings.

Highlights

- Xpert® CT/NG and TV assays can detect STIs in resource-constraint settings.
- Logistical challenges (temperature, transport time) may affect Xpert® performance.
- *Chlamydia trachomatis* and *Neisseria gonorrhoeae* detection is not affected.
- Small reduction of *Trichomonas vaginalis* detection occurs in urine after two weeks.
- Xpert® detection of STIs is logistically feasibly in resource-constraint settings.

7.1 Manuscript

Sexually transmitted infections (STIs) caused by *C. trachomatis*, *N. gonorrhoeae* and *T. vaginalis* remain a major health challenge globally and Sub-Saharan Africa has the highest burden of STIs with 98 million infections reported in 2012 (Newman et al., 2015). STIs in Sub-Saharan Africa are treated using a syndromic management approach which has well documented limitations such as the inability to detect asymptomatic infections, lack of antimicrobial susceptibility testing, unnecessary and inappropriate use of antibiotics (overtreatment) with the risk of antimicrobial resistance development, and lack of laboratory infrastructure for surveillance (Garrett et al., 2018, White et al., 2008). Therefore, implementation of routine molecular laboratory diagnostics is imperative in order to address the high burden of STIs in Sub-Saharan Africa.

Traditionally laboratory diagnosis of *C. trachomatis*, *N. gonorrhoeae* and *T. vaginalis* from clinical specimens relies on microscopy and culture methods which are time and labour intensive and have a low sensitivity (Hobbs and Seña, 2013, Jacobsson et al., 2018). The Cepheid GeneXpert® CT/NG and Xpert® TV assays are rapid fully automated molecular assays that have excellent sensitivity (97.5%) and specificity (99.9%) for diagnosing STIs and have been used in various settings (Gaydos, 2014, Peters et al., 2017, Schwebke et al., 2018). These assays can be rolled-out as routine diagnostics in resource-constraint settings by leveraging existing infrastructure of GeneXpert® platforms already in place for the detection of *Mycobacterium tuberculosis* (Parsons et al., 2011).

However, the effectiveness of GeneXpert® assays for STI diagnosis in resource-constraint settings may be undermined by logistical challenges such as the temperature at which specimens are stored and specimen transport time to laboratory facilities (Lu et al., 2016). Our aim was to determine the effects of storage temperature and transport time on the detection of *C. trachomatis, N. gonorrhoeae* and *T. vaginalis* infections using the GeneXpert® platform in a real-life setting in Pretoria, South Africa. The manufacturer recommends collection and storage of specimens for Xpert® processing using the urine specimen collection kit (for Xpert® CT/NG testing male urine is considered stable at 2-30°C for 45 days and female urine up to 45 days at 2°C to 15°C and for 3 days up to 30°C in this kit; for processing by the Xpert® TV assay urine is considered stable up to 14 days at 30°C and the endocervical specimen collection

kit (stable at 30°C for up to 60 days for Xpert® CT/NG as well as Xpert® TV testing). To our knowledge, there has not been an independent evaluation of these specimen collection kits when used in practice in settings in sub-Saharan Africa.

To investigate the impact of specimen storage temperature and transport time for detection of *C. trachomatis* and *N. gonorrhoeae* using the Xpert CT/NG test we created a stock solution by repeated centrifugation followed by suspension of the pellet in 2 mL phosphate saline buffer (PBS) (Merck, Germany) of a known *C. trachomatis*-positive urine sample (quantified using the Light mix Kit 480 HT CT/NG assay (TIB MOLBIOL, Berlin, Germany) on the LightCycler® (Roche Diagnostics, Switzerland) that was spiked with a stock culture of *N. gonorrhoeae* ATCC® 49226 strain based on viable count. Three 10-fold dilutions of this stock solution were prepared, and these were spiked to urine and PBS. Aliquots of these spiked stock dilutions were then transferred to the Xpert® urine specimen collection (6 mL) and the Xpert® endocervical specimen collection (0.25 mL) kits in line with the manufacturer's instruction (Cepheid, Sunnyvale, USA). Thereafter, the transport collection kits were either stored at room temperature (27°C ± 3°C) which is normal summer room temperature for our setting in Pretoria, South Africa, or kept in the refrigerator (4°C± 1°C).

One millilitre of sample from each stored Xpert® specimen collection kit was transferred to the Xpert® CT/NG reaction cartridge resulting in a final cycle threshold (Ct) value range of 25-34 for *C. trachomatis* and 10, 000 -100 colony forming units for *N. gonorrhoeae* for the 10-fold dilution series in the Xpert® test reaction. Xpert® CT/NG test was performed on two samples from each specimen transport medium at days 0, 7, 14 and 28 for detection of presence of *C. trachomatis* and *N. gonorrhoeae* DNA. We chose a series of up to 28 days as realistically specimens should be processed within that time frame to have clinical value in routine practice. The same procedure was followed for analysis of *T. vaginalis*; filter sterilised urine (6 mL) that was spiked with a counted stock solution of *T. vaginalis* ATCC® 30001 strain was transferred to the Xpert ® specimen transport kits resulting in a concentration range of 11000-1100 organisms per millilitre in the kits. From these kits 0.5 mL was transferred to the Xpert® TV reaction cartridges corresponding to 5,500-55 protozoal cells per Xpert® test reaction. The Ct values provide a semiquantitative concentration of DNA in the sample and a change in Ct value of more than 2 cycles for the spiked samples was considered clinically relevant (Ingersoll et al.,

2008). Specimens from the different time points were concurrently processed and tested in the same run.

We did not observe any clinically relevant increase in the Ct value over time between baseline (day 0) and the subsequent time points (days 7, 14, 28) for detection of *C. trachomatis* and *N. gonorrhoeae* spiked in either Xpert® specimen collection kits irrespective of the storage temperature (**Figure 7.1; Table 7.2 Supplementary material A).** However, there was a pronounced increase in the Ct-value of *T. vaginalis* spiked in the Xpert® urine specimen collection kit stored at room temperature (3,5 (13%), 2,6 (8.5%) and 0,45 (1.3%) respectively for the highest, medium and lowest spiking concentration), but not in the refrigerator. The increase in the Ct value from the aliquots of the Xpert® urine specimen collection kit stored at room temperature (27°C ± 3°C) correlates to a reduction in *T. vaginalis* DNA load of ~10 fold. There were no differences observed for detection of *T. vaginalis* from the endocervical collection kit.

This study has several limitations. First, specimens were not actually transported but left at room temperature (27°C ± 3°C) in a single venue with its temperature fluctuations during the summer months in Pretoria, South Africa. In practice, the delay in sample processing with fluctuations in temperature over time would most likely occur from storage in a single place rather than continuously for a longer period of time. As such, we do not think that this really influenced our results. To mimic what would happen in routine practice in a sub-Saharan setting, we decided to keep a realistic scenario for our setting, i.e. using the normal room temperature (27°C ± 3°C) during summer instead of artificially modifying temperature height and fluctuations. This might have biased the results, in particular, in relation to detection of *T. vaginalis* from urine, however, we do think it is important to test specimen storage conditions in a realistic manner as to inform guideline and policy design. In practice, simple solutions such as a foam/cooler box without ice could be implemented to prevent specimens being exposed to temperatures higher than recommended and evaluated in our study. We used PBS for spiking instead of endocervical fluid for spiking of the endocervical specimen collection kit. The absence of vaginal microbiota that would normally have been present in specimens and might affect DNA integrity and stability might have biased our results. Finally, we only included a single strain of each microorganism, evaluated a dual rather than single positive for *C.*

trachomatis and *N. gonorrhoeae*, and used filtered sterilised urine for spiking of microorganisms, but have no reason to think that this has influenced our results.

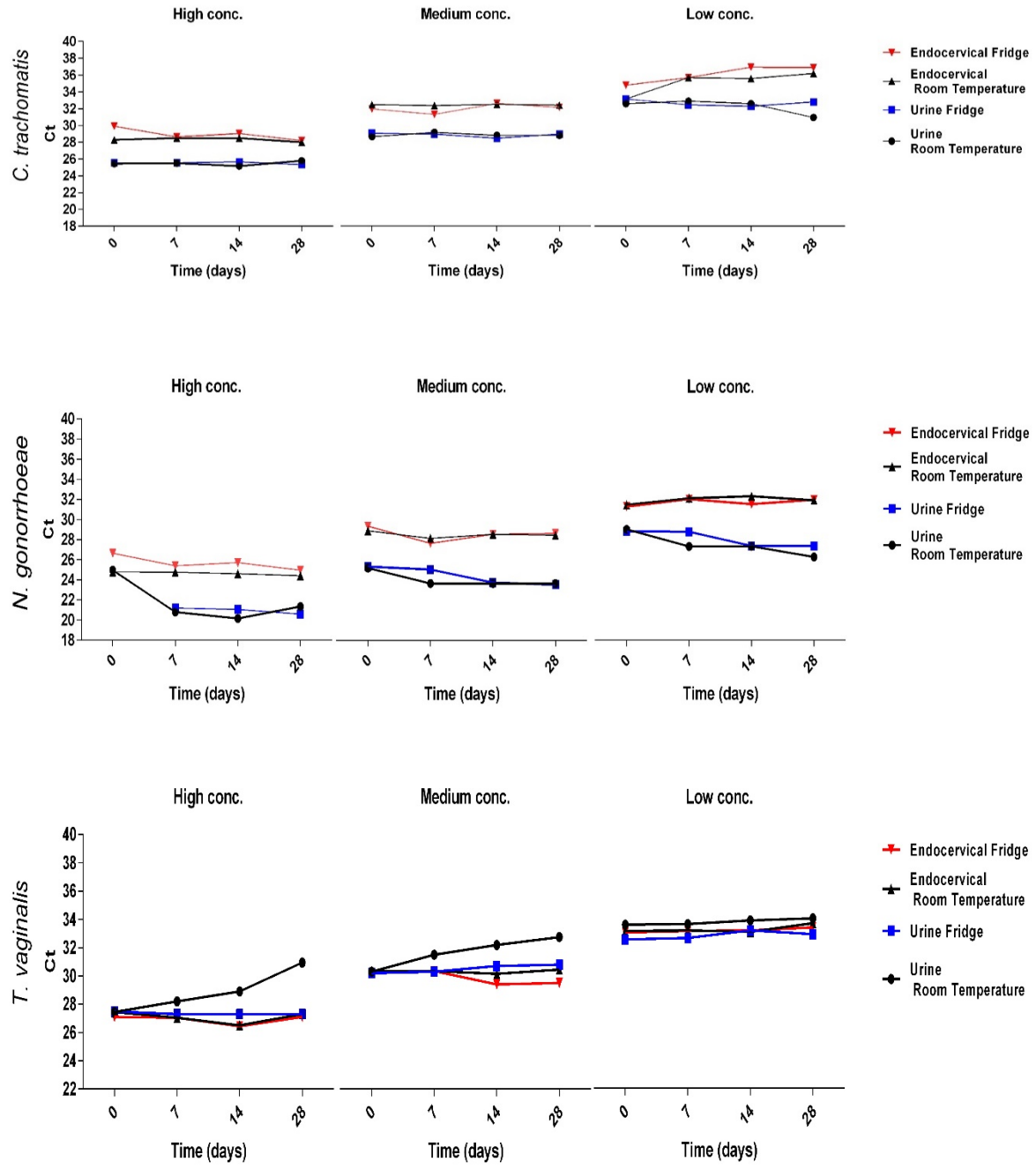

Figure 7.1 Mean cycle threshold values for *Neisseria gonorrhoeae*, *Chlamydia trachomatis* and *Trichomonas vaginalis* spiked to the Xpert® endocervical and urine specimen collection kits for each time point and different storage conditions.

Chlamydia trachomatis, *N. gonorrhoeae* and *T. vaginalis* remain important STI pathogens in Sub-Saharan Africa where these infections are managed using a syndromic approach and absence of routine laboratory diagnostics (Garret et al 2008). Our data show that genital specimens stored in transport medium at temperatures of up to 30°C and processed within 28 days from collection provide reliable results in Xpert® CT/NG testing. However, we observed a small decrease in detection of *T. vaginalis* DNA from the Xpert urine collection kit specimens at room temperature (27°C ± 3°C). This suggests that specimens being tested for *T. vaginalis* should preferably be refrigerated to avoid a potential small reduction in test sensitivity. This observation is confirmed by Ingersoll et al (Ingersoll et al., 2008) who reported better recovery of *T. vaginalis* DNA when specimens were refrigerated. Our data confirm the specifications made by the manufacturer in the package insert, however, we are not aware of any other evaluations of these specifications. Moreover, we used an experimental set-up that translates to implementation of routine diagnostics in practice in sub-Saharan Africa and confirm that this would be feasible using the Xpert® collection media with subsequent laboratory STI testing. A simple solution to keep the specimens at recommended temperature would be to use a foam or cooler box, without an ice requirement, to keep specimens at an appropriate temperature.

In summary, our work highlights that logistical barriers associated with specimen storage temperature at 30°C and transport time of up to 28 days may not impact the implementation of STI diagnostics using existing GeneXpert® platform in resource-constraint settings.

7.2 Declaration of Completing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

7.3 Acknowledgements

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Table 7.1 Supplementary data A. Cycle threshold values for *Neisseria gonorrhoeae, Chlamydia trachomatis* and *Trichomonas vaginalis* spiked to the Xpert endocervical and urine specimen collection kits

Time Point (day)	Urine Room temperature (Ct)			Urine Fridge (Ct)			Endocervical swab room temperature (Ct)			Endocervical swab fridge (Ct)		
	Sample 1	Sample 2	Mean	Sample 1	Sample 2	Mean	Sample 1	Sample 2	Mean	Sample 1	Sample 2	Mean
***Chlamydia trachomatis* high concentration**												
0	25,7	25,2	25,45	25,8	25,3	25,55	28,3	28,3	28,3	31,2	28,6	29,9
7	25,8	25,2	25,5	25,6	25,5	25,55	28,7	28,3	28,5	28,5	28,8	28,65
14	25,2	25,1	25,15	25,6	25,7	25,65	28,5	28,5	28,5	29	29,1	29,05
28	26,1	25,5	25,8	25,1	25,6	25,35	27,9	28,1	28	28,1	28,3	28,2
***Chlamydia trachomatis* medium concentration**												
0	28,6	28,7	28,65	29	29,1	29,05	32,2	32,7	32,45	31,7	32,2	31,95
7	29,2	29,1	29,15	29	28,9	28,95	32,2	32,5	32,35	32,5	30,1	31,3
14	29,3	28,3	28,8	28,7	28,2	28,45	32,5	32,5	32,5	32,5	32,7	32,6
28	28,7	28,9	28,8	29	28,9	28,95	32,2	32,6	32,4	31,9	32,4	32,15
***Chlamydia trachomatis* low concentration**												
0	32,7	32,5	32,6	33,3	33	33,15	33,3	33	33,15	35,5	34,1	34,8
7	32,7	33,1	32,9	32,7	32,2	32,45	35,3	36,1	35,7	35,4	36	35,7
14	32,3	32,9	32,6	32	32,6	32,3	35,2	36	35,6	36,3	37,6	36,95
28	30,8	31,1	30,95	32,9	32,7	32,8	36,1	36,3	36,2	37,2	36,6	36,9

Table 7.1 Supplementary data A. Cycle threshold values for *Neisseria gonorrhoeae, Chlamydia trachomatis* and *Trichomonas vaginalis* spiked to the Xpert endocervical and urine specimen collection kits (Continued)

Time Point (day)	Urine Room temperature (Ct)			Urine Fridge (Ct)			Endocervical swab room temperature (Ct)			Endocervical swab fridge (Ct)		
	Sample 1	Sample 2	Mean	Sample 1	Sample 2	Mean	Sample 1	Sample 2	Mean	Sample 1	Sample 2	Mean
***Neisseria gonorrhoeae* high concentration**												
0	24,9	25	24,95	21,2	21	21,1	24,8	24,8	24,8	28,2	25,1	26,65
7	21,2	20,4	20,8	21,3	21,1	21,2	25,1	24,4	24,75	25,2	25,6	25,4
14	20,2	20,1	20,15	21	21,1	21,05	24,5	24,7	24,6	25,3	26,1	25,7
28	21,5	21,2	21,35	20,4	20,8	20,6	24,3	24,5	24,4	24,9	25	24,95
***Neisseria gonorrhoeae* medium concentration**												
0	25,1	25,3	25,2	25,2	25,5	25,35	28,5	29,3	28,9	28,4	30,3	29,35
7	23,4	23,9	23,65	25,1	25	25,05	28,1	28,2	28,15	28,9	26,4	27,65
14	24,1	23,2	23,65	24,3	23,2	23,75	28,4	28,7	28,55	28,6	28,5	28,55
28	23,7	23,6	23,65	23,6	23,5	23,55	28,3	28,6	28,45	28,5	28,8	28,65
***Neisseria gonorrhoeae* low concentration**												
0	29	29,1	29,05	28,8	28,9	28,85	31,5	31,5	31,5	31,3	31,3	31,3
7	27,3	27,4	27,35	28,7	28,9	28,8	32,2	32,1	32,15	32,1	32	32,05
14	27,5	27,2	27,35	27,4	27,4	27,4	32,2	32,5	32,35	31,7	31,4	31,55
28	26,1	26,5	26,3	27,5	27,3	27,4	32,3	31,6	31,95	32,4	31,6	32

Table 7.1 Supplementary data A. Cycle threshold values for *Neisseria gonorrhoeae, Chlamydia trachomatis* and *Trichomonas vaginalis* spiked to the Xpert endocervical and urine specimen collection kits (Continued)

Time Point (day)	Urine Room temperature (Ct)			Urine Fridge (Ct)			Endocervical swab room temperature (Ct)			Endocervical swab fridge (Ct)		
	Sample 1	Sample 2	Mean	Sample 1	Sample 2	Mean	Sample 1	Sample 2	Mean	Sample 1	Sample 2	Mean
***Trichomonas vaginalis* high concentration**												
0	27,5	27,4	27,45	27,6	27,4	27,5	27,7	27,2	27,45	27	27,2	27,1
7	27,8	28,6	28,2	27,3	27,3	27,3	27	27,1	27,05	27,2	26,9	27,05
14	29,1	28,7	28,9	27,4	27,2	27,3	26,3	26,7	26,5	26,5	26,4	26,45
28	30,5	31,4	30,95	27,3	27,3	27,3	27,3	27,3	27,3	27,1	27,1	27,1
***Trichomonas vaginalis* medium concentration**												
0	30,3	30,1	30,2	30,10	30,30	30,2	30,30	30,40	30,35	30,30	30,10	30,2
7	31,2	31,8	31,5	30,40	30,20	30,3	30,40	30,30	30,35	30,40	30,60	30,5
14	32,1	32,3	32,2	31,20	30,20	30,7	30,50	29,80	30,15	29,60	29,20	29,4
28	33,10	32,40	32,75	30,20	31,40	30,8	30,50	30,40	30,45	29,20	29,80	29,5
***Trichomonas vaginalis* low concentration**												
0	33,2	34	33,6	32,6	32,5	32,55	33,2	33,1	33,15	33,3	32,8	33,05
7	33,7	33,6	33,65	32,9	32,4	32,65	33,2	33,2	33,2	33,3	33	33,15
14	33,7	34,1	33,9	33,4	33	33,2	33,3	32,9	33,1	33,3	33,1	33,2
28	34,3	33,8	34,05	32,9	Invalid	32,9	33,40	34,00	33,7	33,60	33,20	33,4

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CHAPTER 8

Concluding Discussion

Neisseria gonorrhoeae remains a major public health priority globally with an estimated 87 million infections in 2016 (WHO, 2018). Since the advent of antimicrobial therapy in the 1930s, *N. gonorrhoeae* has rapidly developed resistance to previously and currently recommended antimicrobials for its treatment (Unemo *et al.*, 2016; Wi *et al.*, 2017). Antimicrobial resistance in *N. gonorrhoeae* first emerged and spread in core transmission groups such as men who have sex with men (MSM) and men with repeat urethral discharge (Lewis, 2013). The emergence of *N. gonorrhoeae* strains showing resistance to presently recommended antimicrobials (ceftriaxone plus azithromycin) and the dwindling pipeline of new antimicrobials signals the prospect of untreatable gonorrhoea is near (Fifer *et al.*, 2016; Wi *et al.*, 2017; Unemo *et al.*, 2019b). In response to this threat, the WHO recently published its first ever list of antibiotic-resistant "priority pathogens" and has ranked antimicrobial resistant *N. gonorrhoeae* priority's tier, as high (Tacconelli *et al.*, 2017). In addition, the CDC has declared emergence of drug-resistant *N. gonorrhoeae* one of the top three global AMR urgent threats to public health (CDC, 2019). To address the rising threat of antimicrobial resistant *N. gonorrhoeae* the United Nations (UN) World Health Assembly in 2016 endorsed the WHO global health sector on Sexually Transmitted Infections (STIs), that outlines a 90% reduction in gonorrhoea incidence target by 2030 (WHO, 2016). To reduce the incidence of gonorrhoea, it is imperative to mitigate the rising threat of antimicrobial resistance in *N. gonorrhoeae.* Strengthened *N. gonorrhoeae* antimicrobial resistance surveillance is crucial to identify underlying drivers of transmission of antimicrobial resistant infections and to provide evidence for revisions of gonorrhoea national treatment guidelines, as well as formulations of public health policy guidelines (Alirol *et al.*, 2017).

Mycoplasma genitalium infection frequently occurs as co-infection with other STIs in high-risk populations (Marrazzo and Holmes, 2014). Untreated *M. genitalium* infection is associated with various reproductive complications (Unemo and Jensen, 2017). The efficacy of doxycline which was used for prolonged periods of time for treatment of *M. genitalium* has dwindled and has led to the use of azithromycin as preferred treatment (Couldwell and Lewis, 2015). However, the rising incidence and emerging reports of antimicrobial resistance in *M. genitalium* is of major public health concern (Sethi *et al.*, 2017). Azithromycin treatment failure was first

reported in Australia and has subsequently emerged globally (Couldwell and Lewis, 2015). Emergence of macrolide resistant *M. genitalium* is directly linked to (over-) consumption of antibiotics and now threatens the provision of effective therapy (Couldwell and Lewis, 2015; Unemo and Jensen, 2017). Currently, the emergence of MDR *M. genitalium* strains associated with treatment failures is of significant public health concern due to associated complications (Sethi *et al.*, 2017). Fluoroquinolones such as moxifloxacin, gatifloxacin and pristinamycin remain highly effective against macrolide resistant *M. genitalium* However, cases have been documented of moxifloxacin treatment failure, due to *M. genitalium* strains with coexistent macrolide-associated and fluoroquinolone-associated mutations (Dionne-Odom et al., 2017). Continual inappropriate antimicrobial stewardship in the use of newer antibiotics will likely lead to untreatable *M. genitalium* infections in the future.

8.1 General summary of findings

The purpose of this PhD was to investigate the phenotypic and genotypic antimicrobial resistance in two core transmission groups of men in South Africa. The results from the present study (Chapter 3) confirmed that a population with high-risk sexual behaviour was recruited; most participants reported unprotected sex, multiple sex partners, coinfection rate with other STIs was high and had a recent history of treatment for STIs. The introduction of routine diagnostics in clinical management and prompt treatment to reduce transmission is warranted in this high-risk population with a high burden of *N. gonorrhoeae* infection. *N. gonorrhoeae* infection was the predominant cause of the male urethritis syndrome (82%) in our study. The importance in *N. gonorrhoeae* infection is supported by studies that revealed a high burden of *N. gonorrhoeae* infection in similar MSM populations, as well as men in the general population (Mhlongo *et al.*, 2010; Rebe *et al.*, 2015; Kularatne *et al.*, 2018; Van Liere *et al.*, 2019).

High rates of antimicrobial resistance were detected among gonococcal isolates collected from both the public healthcare sector (Chapter 3) and the private healthcare sector (Chapter 6). Most of the gonococcal isolates showed resistance to tetracycline and ciprofloxacin, with one-third of these isolates displaying a resistance profile that can be classified as being multidrug-resistant (MDR). The high rates of *N. gonorrhoeae* drug resistance observed in this PhD study support the trends reported in the national surveillance data and by other studies in South Africa (Lewis *et al.*, 2008; Kularatne *et al.*, 2018; Rambaran *et al.*, 2019). Tetracycline and ciprofloxacin were extensively used in the syndromic management for STIs in South Africa for a prolonged period of time and it is unlikely that these can be repurposed (Lewis *et al.*, 2008;

Kularatne *et al.*, 2018). The escalation in the prevalence of ciprofloxacin resistance in Durban, Johannesburg and Cape Town prompted to its abandonment for treatment of gonorrhoea in 2008 (Moodley and Sturm, 2004; Lewis *et al.*, 2008). Despite its abandonment in treatment of gonorrhoea, high rates of ciprofloxacin-resistant *N. gonorrhoeae* remain. This could be attributed to the continued use of ciprofloxacin in both the private and public healthcare sector for various indications including male dysuria as per current standard treatment guidelines. The high rates of tetracycline resistance could be attributed to the use of doxycline in syndromic management of non-gonococcal urethritis until five years ago in South Africa when STI management guidelines were revised (Department of Health, 2015). Unexpectedly, relatively high rates of penicillin resistance (89%) were observed from gonococcal isolates from the private healthcare sector, but not the public healthcare sector, considering that the national sentinel surveillance reported penicillin resistance prevalence of 51% (Kularatne *et al.*, 2018). Penicillin is one of the β-lactam classes of antibiotics which are commonly prescribed for a variety of clinical indications (Pandey and Cascella, 2019). These findings might be explained by the selection pressure from the use of amoxicillin and amoxicillin/clavulanic acid in the private healthcare sector for unrelated infections such as respiratory tract infections and urinary tract infections. These results highlight the importance of including gonococcal isolates from the private healthcare sector in future national sentinel surveillance.

Azithromycin is currently used to treat gonococcal infections in combination with ceftriaxone in many countries and was only introduced in 2015 in the syndromic management of urethral discharge in South Africa (Department of Health, 2015). In this PhD study, a relatively high rate of azithromycin resistance among gonococcal isolates was observed considering its recent introduction in the syndromic management regimen, although this observation is based on a relatively small sample size of a selected core transmission group of men. These findings suggest azithromycin resistance in *Neisseria gonorrhoea* could be emerging in South Africa. This is supported by the recent data from primary healthcare centres in Durban and Pietermaritzburg; Kwazulu-Natal province where azithromycin resistance was detected in 68% of the isolates (Rambaran *et al.*, 2019). However, this is in stark contrast to data from the national sentinel surveillance reporting azithromycin resistance of 5% (Kularatne *et al.*, 2018). The discrepancies in the results between these studies could be that the data presented in the national sentinel surveillance included only a limited number of facilities and core transmission groups were not included in the surveillance. In addition, the surveillance data covered a 10-

year period (2007-2017) and azithromycin resistance had not yet emerged as it was only introduced in 2015 in the syndromic management regimen (Kularatne *et al.*, 2018).

There was a good correlation between previously described *N. gonorrhoeae* genetic resistance mutations and the phenotypic antimicrobial susceptible profiles for the antimicrobials tested (Unemo, 2019a) Whole genome sequencing analysis revealed azithromycin resistance in this study was due to mutations in the *mtrR* promoter (-35A del) and A39T in the MtrR repressor. The observed azithromycin resistance of *N. gonorrhoeae* in core groups warrants an urgent clarification of what is happening in South Africa. High rates of azithromycin resistance may have implications for the syndromic management guidelines. Azithromycin was introduced as a component of dual therapy in combination with ceftriaxone to slow down development and spread of gonococcal resistance to extended spectrum cephalosporins (Rice, 2015). In addition, using both ceftriaxone and azithromycin as dual therapy for gonorrhoea could result in synergy between the two antibiotics, thereby increasing potency (Barbee *et al.*, 2014; Mensforth and Ross, 2019). Azithromycin resistance effectively results in monotherapy with ceftriaxone; this could select for cephalosporin resistance in *N. gonorrhoeae* and thus the emergence of ceftriaxone resistant gonococcal strains. However, exclusion of azithromycin from the syndromic management regimen in South African healthcare sector might be challenging without introduction of diagnostics for management of STIs, as azithromycin covers other STIs (*M. genitalium* and *C. trachomatis*), which commonly occur as a co-infection with *N. gonorrhoeae.* An alternative to azithromycin in the syndromic management of gonorrhoea could be doxycline which also has efficacy against *C. trachomatis* and *M. genitalium* infections.

The present study is one of the first studies in Africa to provide in-depth genomic analysis of *N. gonorrhoeae* infection among core transmission groups. WGS analysis of the gonococcal isolates revealed a wide diverse epidemic of *N. gonorrhoeae* strains circulating in South Africa based on the high number of novel sequence types (STs) identified. A substantial number of novel *N. gonorrhoeae* sequence typing for antimicrobial resistance (NG-STAR) (19/27;70%) and *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) (19/27; 70%) STs, which have not been reported elsewhere in the world, were identified. The identification of a substantial number of novel *N. gonorrhoeae* sequence types highlights the importance of including gonococcal strains from resource-constraint settings when evaluating new treatment regimens or antimicrobials as gonococcal strains could have different genetic resistance mutations from gonococcal strains from well-resourced settings.

Mycoplasma genitalium infection frequently occurs as co-infection with other STIs in high-risk populations (Marrazzo and Holmes, 2014). *Mycoplasma genitalium* co-infection with other STIs could result in synergy in transmission of the pathogen and disease severity (Getman *et al.*, 2016; Alirol *et al.*, 2017; Fernández-Huerta and Espasa, 2019). *Mycoplasma genitalium* has developed resistance to macrolides and fluoroquinolones at alarming rates globally (Couldwell and Lewis, 2015; Sethi *et al*., 2017). Antimicrobial resistance has compromised treatment of *M. genitalium* infections and treatment failures have been reported worldwide including Sub-Saharan Africa, where syndromic management is used (Couldwell and Lewis, 2015; Hay *et al.*, 2015; Le Roux *et al.*, 2018). In South Africa, *M. genitalium* infection is covered by azithromycin in the syndromic management regimen. Higher rates of antimicrobial resistant *M. genitalium* infections were detected from the specimens collected in the private healthcare sector (Chapter 6) than in the public healthcare sector (Chapter 4 and 5). Mutations in the 23S rRNA at position 2071 and 2072 (*M. genitalium* numbering) associated with macrolide resistance were common among isolates collected from the private healthcare sector compared to the public healthcare sector. In addition, various amino acid alterations in the ParC and GyrA of the quinolone resistance determining regions associated with fluoroquinolone resistance were detected. These findings suggest that macrolide resistant *M. genitalium* is emerging in the private health care sector in South Africa, whereas levels appear to remain low in the public sector for now. This could be attributed to the use of azithromycin for unrelated indications such as upper respiratory infections in the private healthcare, as development of antimicrobial resistance in *M. genitalium* is directly linked to (over-) consumption of the related class of antibiotics (Dong and Klausner, 2019; Kenyon *et al.*, 2019). Molecular epidemiological analysis showed wide genetic diversity among the *M. genitalium* isolates. The results from the present study confirmed that antibiotic resistant *M. genitalium* infections have the potential to undermine the effectiveness of syndromic management of STIs. In the context of syndromic management, diagnostics and molecular susceptibility testing for *M. genitalium* is not available. In addition, the current national sentinel surveillance in South Africa focuses on AMR in *N. gonorrhoeae* and is limited to public sector facilities. Symptomatic infection would be covered by azithromycin; however, treatment failure cases due to macrolide-resistant *M. genitalium* are unlikely to be detected in the absence of routine diagnostics. In addition, moxifloxacin used for the treatment of macrolide-resistant *M. genitalium* infection is not included in the current syndromic management algorithm nor routinely available in the public healthcare system in South Africa.

An important intervention to combat the high burden of drug-resistance in *N. gonorrhoeae* infections is the introduction of molecular laboratory diagnostics in clinical management of STIs to allow for targeted antimicrobial treatment. However, logistical challenges such as the temperature at which the specimens are stored and specimen transport time to laboratory facilities may potentially undermine the effectiveness of molecular diagnostic tests. The effects of specimen storage and transport time on the detection of *C. trachomatis*, *N. gonorrhoeae* and *T. vaginalis* infections using the GeneXpert® platform in resource constraint settings were investigated (Chapter 7). The findings suggest that specimens stored in Xpert® transport medium at room temperature (27°C ± 3°C) and processed within one month provide reliable results for detection of *C. trachomatis* and *N. gonorrhoeae* using the GeneXpert® platform. However, there was a small reduction in detection of *T. vaginalis* DNA after two weeks and therefore *T. vaginalis* specimens should preferably be kept in the refrigerator. This study demonstrated that logistical challenges of specimen storage, temperature and transport time are unlikely to impact on performance of routine STI diagnostics using the GeneXpert® platform implemented in resource-constraint settings. This information is useful to policy makers in implementation of the GeneXpert® platform for routine diagnostics in resource-constraint settings where there is a high burden of STIs and where these infections are currently managed using a syndromic management approach.

There were some limitations noted in the present PhD study. A relatively small sample size for the study cohorts in both the public healthcare and private healthcare sectors was one of the limitations. Mobilization and recruitment of core groups was challenging despite intensified efforts to identify potential study participants. The low numbers of participants that were recruited reflects on the challenges of obtaining specimens from core groups in the South African public health setting. This is due to cultural issues such as the stigma associated with same-sex sexual relationships. Furthermore, in a clinical setting where routine diagnostics is not part of clinical practice there is generally a lack of awareness among the healthcare professionals regarding the importance of STI surveillance programmes and therefore it is viewed as simply a special research project. Participant recruitment could be affected as result because healthcare professionals may view collecting specimens and demographic data from patients as an extra duty because current standard of care is the syndromic management of STIs. However, despite the low number of participants, the present study provided a comprehensive overview of *N. gonorrhoeae* and *M. genitalium* infections with a concerning message of emerging drug resistance. Lastly *N. gonorrhoeae* culture viable isolates were recovered in two-

thirds of the specimens with a positive molecular test. The result shows some of the logistical challenges of obtaining specimens from a primary healthcare centre and transporting these to the laboratory, which sometimes takes several days for the specimens to reach the laboratory in resource-constraint settings.

8.2 Perspectives in improving STI control in South Africa

Neisseria gonorrhoeae remains a major public health concern globally, with one of highest incidence of gonorrhoea found in Sub-Saharan Africa (Alirol *et al.*, 2017). Despite this, there is very limited data of *N. gonorrhoeae* strains circulating in Africa and their mechanisms of resistance. This paucity of data is due to weak surveillance systems in many African countries, the absence of routine diagnostics, and the use of syndromic management for STIs (Ndowa *et al.*, 2013). Core transmission groups such as MSM and those with recurrent gonorrhoea episodes have played an important role in the emergence and spread of drug resistance. The advent of whole genome sequencing has provided robust evidence of current gonococcal AMR burden and the underlying molecular mechanisms of AMR. However, such studies have focused on gonococcal strains from high-income countries (De Silva et al., 2016; Demczuk *et al.,* 2017). Conducting quality-assured *N. gonorrhoeae* antimicrobial resistance surveillance studies using WGS in resource-constraint settings is challenging. This is due to limited resources, absence of routine laboratory diagnostics, logistical issues such as specimen transport time to the laboratory for culture and cultural issues targeting core transmission groups.

In the present PhD study, a comprehensive in-depth WGS analysis of *N. gonorrhoeae* infection among high-risk men was conducted. Gonococcal infection and AMR were highly prevalent in this selected population of high-risk men. The burden of gonococcal AMR will be mitigated when the prevalence of gonorrhoea is reduced. Therefore, there is an urgent need to strengthen sexual health services in South Africa through cost-effective control programmes of STIs, given their synergy with HIV. This can be achieved through novel ways of thinking aspects regarding STI control strategies such as; (1) strengthening the national STI service delivery across all primary healthcare centres and adherence to national STI management guidelines by healthcare professionals, (2) introduction of routine diagnostics for STI aetiological management, (3) introduction of molecular resistance guided therapy in high-risk populations and those that fail treatment, and (4) strengthening the aetiological STI and antimicrobial surveillance systems.

In South Africa, the syndromic management approach for STIs has been implemented for over two decades (Crowther-Gibson *et al.*, 2011). A mathematical modelling study has shown a substantial reduction in the prevalence of curable STIs that are symptomatic, since the introduction of syndromic management for STIs (Johnson *et al.*, 2011). Moreover, the use of syndromic management approach has completely changed the aetiology of genital ulcer syndrome with substantial reduction in the prevalence of chancroid and donovanosis (Johnson *et al.*, 2011). One of the advantages of this approach is provision of same-day treatment for the patients using treatment algorithms and it can easily be followed by the nursing staff across the primary healthcare centres. The syndromic management approach works better in symptomatic STI syndromes in males compared to STI syndromes in women (Crowther-Gibson *et al.*, 2011). Although the syndromic management approach has been implemented and used, there are remaining structural barriers to provision of quality STI service delivery. Barriers such as frequent stockouts of antibiotics and condoms, limited integration of STI and HIV services especially for high-risk populations, inadequate adherence to STI guidelines by healthcare professionals and their attitudes to providing services to key populations (Kohler *et al.*, 2017) need to be addressed to achieve the goal of controlling STIs.

Laboratory diagnostics are crucial in detecting the STI pathogens and characterising AMR strains in patients that fail treatment. Therefore, implementation of routine laboratory diagnostics in our settings to guide therapy for both the index patient and partner followed by a test-of-cure is urgently needed and would require a collaborative action between all stakeholders in the healthcare sector to provide policy and resources. Rolling-out of routine molecular laboratory diagnostics in both public and private healthcare sectors would also provide much needed data for enhancing STI surveillance programmes locally. Such STI surveillance programmes should include collection of data from high-risk populations such as MSM, commercial sex workers (CSW) and transgender individuals, as well as understanding the burden of extra-genital infections common in these groups. Among a plethora of molecular diagnostics assays that are available for detection of curable STIs, the Cepheid GeneXpert® CT/NG and Xpert® TV have excellent sensitivity and specificity for diagnosing STIs and were evaluated in this PhD study (Gaydos, 2014, Schwebke et al., 2018). Currently the GeneXpert® platform does not include the detection of *M. genitalium*; however, the ResistancePlus® MG assay for detection of *M. genitalium* and macrolide resistance associated markers is in the pipeline. With the existing GeneXpert® infrastructure already in place for rapid detection of *Mycobacterium tuberculosis* in South Africa, this can be used to roll-out additional routine

molecular diagnostics for STIs (Garrett *et al.*, 2017). Furthermore, implementation of routine laboratory diagnostics in a clinical setting will help in antimicrobial stewardship through targeted treatment and reduction in over prescribing of antimicrobials for unrelated indications (Ong *et al.*, 2019).

Antimicrobial resistance among STI pathogens is increasing at alarming rates globally and we are facing the threat of untreatable STIs in the future. To address the burden of AMR among STIs, AMR surveillance systems are critical in identifying emerging resistant strains, monitoring the local AMR trends and to provide evidence for revision on national STI management guidelines. The WHO guidelines recommend that empiric treatment should be discontinued when the rates of AMR and therapeutic failures are 5% using data from recent quality-assured AMR surveillance (WHO, 2012). The WHO recommends the use of syndromic management for STIs in resource-constraint settings without adequate laboratory infrastructure, supported with regular quality-assured AMR surveillance. However, due to increasing threats of AMR the WHO also recommends a shift from syndromic management to the aetiological STI detection approach. In South Africa, AMR surveillance is in the context of antimicrobial resistant *N. gonorrhoeae* infections and is limited to research settings and sentinel primary health care facilities (Garrett *et al.*, 2017; Kularatne *et al.*, 2018). South Africa is one of the few countries in Africa participating in the WHO Global Gonococcal Antimicrobial Surveillance Program (GASP) (Unemo and Dillon, 2014; Kularatne *et al.*, 2018). The current surveillance programme has several limitations; it is largely aimed at the heterosexual population with most patients from ante-natal clinics, does not include isolates from the private healthcare sector and an in-depth bio-behavioural data are not collected from participants. To address the emerging threat of antimicrobial resistant *N. gonorrhoeae* infections locally there is a need to strengthen and scale-up the AMR surveillance in South Africa. The strengthened surveillance programme should include high-risk populations and isolates from the private healthcare sector. Historically core groups such as MSM have contributed to emergence and spread of antimicrobial resistance in *N. gonorrhoeae* (Lewis, 2013). Core groups are characterised by a high number of sexual partner change and can transmit *N. gonorrhoeae* and other STIs to a large number of sexual partners (Lewis, 2013). Therefore, AMR surveillance, prompt treatment and understanding gonococcal transmission dynamics among high-risk populations is crucial for management and control of gonorrhoea. Additional investment and resources would be required for enhanced AMR surveillance in these high-risk populations and this would include monitoring treatment failures, detection of extra-genital (pharyngeal) infections and

implementation of test-of-cure (TOC) among high-risk populations. To better understand the burden of AMR STIs across the African continent, a strong collaborative partnership between the countries is needed to build up a strong surveillance network in Africa to facilitate early detection of resistant strains and better inform public health guidelines and policies.

Neisseria gonorrhoeae AMR has emerged to ceftriaxone plus azithromycin, the current first line therapeutic agents (Fifer *et al.*, 2016; Unemo *et al.*, 2019a). One of the key strategies in preservation of antibiotics is the adoption of appropriate antimicrobial stewardship programmes especially for last-line antimicrobials. Implementation of molecular susceptibility-guided antimicrobial stewardship strategies are warranted to address AMR *N. gonorrhoeae* (Ong *et al.*, 2019). Such strategies should: (1) identify aetiological agents of STIs using molecular diagnostics to ensure prompt treatment, reduce over-consumption of antibiotics for unrelated infections and to break the transmission cycle; and (2) to perform molecular antimicrobial susceptibility testing using a near-patient diagnostic assay to enable use of previously abandoned antimicrobials for empiric treatment. Resistance-guided therapy (RGT) for gonorrhoea has been proposed as one strategy in reducing the consumption of ceftriaxone; whereby a diagnostic test is used to determine the molecular susceptibility or resistance at the patient level to guide therapy (Trembizki *et al.*, 2016). Repurposing of ciprofloxacin has been shown to been to be an excellent candidate antibiotic for individualised treatment approach; it is highly efficacious as a single oral dose for sensitive gonococcal strains and well tolerated by patients with few side-effects (Trembizki *et al.*, 2016). However, repurposing of ciprofloxacin in South Africa might not be currently feasible due to high resistance rates but could be potential option for other African countries depending ciprofloxacin resistance rates. Currently, several diagnostics assays for accurate detection of ciprofloxacin resistance have been developed (Ong *et al.*, 2019). These assays detecting key mutations in the GyrA (S91 and D95) associated with ciprofloxacin resistance have shown high sensitivity (98.6%) and specificity (91.4%) (Buckley *et al.*, 2015; Allan-Blitz *et al.*, 2016; Hall *et al.*, 2019). Moreover, mathematical modelling has shown using point-of-care tests to identify ciprofloxacin-sensitive strains can significantly reduce ceftriaxone use (Zienkiewicz *et al.*, 2019). However, the current diagnostic assays developed for predicting azithromycin, cefixime and ceftriaxone resistance are less adequate for prediction of AMR in *N. gonorrhoeae,* as there is a suboptimal correlation between the phenotypic antimicrobial testing and genotype (Deng *et al.*, 2019; Hall *et al.*, 2019; Ong *et al.*, 2019). On a similar note, *M. genitalium* infections are now becoming increasingly difficult to treat due to the emergence of macrolide and fluoroquinolone resistant strains. High rates of

azithromycin resistance in *M. genitalium* strains from the private healthcare sector were observed in the current PhD study. Implementation of resistance guided therapy is urgently warranted in the private healthcare sector. Resistance guided therapy for *M. genitalium* is a three-step strategy that enables individualised treatment according to the macrolide resistance profile. This strategy reduces the use of azithromycin for treatment of *M. genitalium* through: (1) empiric treatment with doxycline, (2) identification of *M. genitalium* coupled with resistance testing and (3) switch of therapy to azithromycin or fluoroquinolone based on resistance testing results (Durukan *et al.*, 2019).

8.3 Future perspectives

Sexually transmitted infections cause substantial morbidity and mortality worldwide through their impact on reproductive health, newborn and child health, as well as facilitating the transmission of HIV (Mayaud and Mabey, 2004). Since the emerge of HIV, STIs have been largely neglected as a public health priority and STI control efforts have been in the context of reducing the risk HIV transmission (Low et al., 2006). However, STIs other than HIV are important public health priorities on their own right, but currently the available funding for implementation of STI control are inadequate. Global funding for STI control programmes (investment of routine diagnostics and enhanced STI surveillance) needs to be mobilised and such funding should not be in the context of HIV prevention programmes.

To adequately control *N. gonorrhoeae* infections, new approaches are needed which include prevention, laboratory diagnostics and management. In the short-term pipeline the introduction of routine laboratory diagnostics in selected populations such as high-risk populations is urgently warranted in South Africa to address the burden of STIs. However, there are several challenges with regard to roll-out of routine diagnostics in STI care that would need to be addressed. Most importantly, potential costs and available funding associated with implementation for such a programme requires attention. Therefore, a concerted effort will be required from the policy makers and healthcare professionals, as well as conducting feasibility studies on the cost-effectiveness. In addition, structural issues must be addressed such as adequate laboratory facilities, available laboratory reagents and enhanced training of laboratory staff. In future, development and implementation of accurate point-of-care (POC) tests at the primary healthcare centres is essential to guide precision treatment and management of *N. gonorrhoeae* and *M. genitalium* infections. Such POC tests should ideally detect the pathogen and perform antimicrobial susceptibility testing. The roll-out of POC tests with simultaneous

antimicrobial susceptibility testing would enable individualised treatment at the first visit to the primary healthcare centre and should include asymptomatic patients such as antenatal and family planning attendees. This would result in the reduction in the selection on the empiric antibiotics and preventing onward transmission of antimicrobial resistant strains.

Non-antimicrobial therapeutic interventions and vaccines are needed to stem the rising threat of antimicrobial resistance of *N. gonorrhoeae* globally. Historically attempts to develop vaccines in *N. gonorrhoeae* have failed due to the antigenic variability of the gonococcus and its propensity to cause multiple infections without inducing protective immunity (Gottlieb *et al.*, 2019). However, a proof-of-principle study has shown promising results in gonococcal vaccine development (Ong *et al.*, 2019). A group B meningococcal outer membrane vesicle vaccine was demonstrated to confer a cross-protection against *N. gonorrhoeae* infection in a New Zealand cohort even though the efficacy was very low (31%) (Petousis-Harris *et al.*, 2017; Gottlieb *et al.*, 2019). Therefore, more research and development efforts are required to find alternative vaccine candidates with better efficacy for *N. gonorrhoeae* infections.

Oropharyngeal infections play an important role in the transmission of AMR *N. gonorrhoeae* (Fairley *et al.,* 2017) especially in high-risk populations such as MSM and CSWs. Several studies have shown gonococcal AMR may evolve in the oropharynx due to horizontal gene transfer from other commensal *Neisseria* spp (Furuya *et al*., 2007; Unemo and Shafer, 2011). The use of mouthwash has been debated as a novel method for gonorrhoea prevention in the oropharynx and is currently being evaluated in several studies (Chow *et al.*, 2017). However, more important research questions needs to be addressed on the practical applicability of using a mouth against gonococcal infections, such as the mouth wash brand that works better than others, the optimum method on how to use the mouth wash (frequency, time, gargling) as well as the timing for its use (daily usage or after-sex).The lack of synergy between azithromycin and ceftriaxone has prompted a debate on the use of azithromycin in dual therapy for gonorrhoea. Other antibiotics to be used in combination with ceftriaxone in dual therapy need to be evaluated as the pipeline for new antibiotics for treatment of gonorrhoea is alarmingly sparse. It is imperative that new drugs are developed and evaluated for treatment of gonorrhoea in future. Zoliflodacin is a new fluoroquinolone that has shown efficacy against urogenital gonococcal infections and is currently being evaluated in phase three clinical trials (Lewis, 2019).

The use of high-throughput sequencing has revolutionised the understanding of the antimicrobial resistance burden and the spread of antimicrobial resistant *N. gonorrhoeae* strains, as well as provide essential surveillance data where there is none. There are important questions regarding antimicrobial resistance that can be addressed using genomics in future such as: (1) the effect of clonal spread of resistant pathogens and *de novo* emergence of resistance and (2) factors influencing the correlation of known genetic resistance mutations and phenotype resistance. In addition, the cost of whole genome sequencing is still prohibitive in resource-constraint settings and there is a need for price decrease over time to make this technology accessible for all settings.

8.4 References

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APPENDIX A Ethical Approval certificates

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 03/14/2020.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

29/06/2017

Approval Certificate
New Application

Ethics Reference No.: 253/2017

Title: Molecular epidemiology and antimicrobial resistance of *Neisseria gonorrhoeae* in men at risk in Gauteng, South Africa

Dear Liteboho Maduna

The **New Application** as supported by documents specified in your cover letter dated 21/06/2017 for your research received on the 23/06/2017, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 28/06/2017.

Please note the following about your ethics approval:
- Ethics Approval is valid for 2 years
- Please remember to use your protocol number (**253/2017**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of **6 monthly written Progress Reports**, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers; MBChB; MMed (Int); MPharMed,PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health).

☎ 012 356 3084 deepeka.behari@up.ac.za / fhsethics@up.ac.za http://www.up.ac.za/healthethics
✉ Private Bag X323, Arcadia, 0007 - Tswelopele Building, Level 4, Room 60, Gezina, Pretoria

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 03/14/2020.

15 May 2019

Approval Certificate
Annual Renewal

Ethics Reference No.: 253/2017
Title: Molecular epidemiology and antimicrobial resistance of Neisseria gonorrhoeae in men at risk in Gauteng, South Africa

Dear Mr LD Maduna

The **Annual Renewal** as supported by documents received between 2019-05-07 and 2019-05-15 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 2019-05-15.

Please note the following about your ethics approval:

- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2020-05-15.
- Please remember to use your protocol number (253/2017) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers
MBChB MMed (Int) MPharmMed PhD
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Research Ethics Committee
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Lefapha la Disaense tša Maphelo

APPENDIX B Data collection forms

A. INFORMED CONSENT FORMS

MEN WITH REPEAT STI; PARTICIPANT'S INFORMATION LEAFLET
&
INFORMED CONSENT FORM

Dear Mr. _____

Date of consent procedure: ……/……/……

This is an informed consent form for men who visit this healthcare facility to consult with the health care worker for symptoms suggestive of sexually transmitted infection (STI). We would appreciate a moment of your time and like to invite you to participate in a research project titled: "**Molecular epidemiology and antimicrobial resistance of *Neisseria gonorrhoeae* in men at risk in Gauteng, South Africa**".

This informed consent form has two parts:

- An information sheet (provides background and useful information about the study)
- A certificate of consent (for signatures if you choose to participate)

Part I: Information sheet

1. Introduction

You are invited to be part of a research project that we are conducting at this healthcare facility to support the treatment of sexually transmitted infections (STIs) in men. Penile discharge is a common symptom that may suggest presence of an STI, but can also be caused other infections. We are specifically looking for men consulting for STI symptoms (penile discharge) that came back or did not disappear following earlier treatment. This information leaflet serves to inform you about the project and to help you to decide if you would like to participate. Before you decide on whether or not you participate, I would like to explain to you why this study is important, why it may be relevant to you, and what participation involves. Before you agree to take part in this study, you should fully understand what is involved. If you have any questions, do not hesitate to ask the investigator. If you agree to participate, you will be asked to sign a consent form.

2. The aim of the study

Sexually transmitted infections (STIs) are infections that are transmitted through sexual contact and may cause symptoms such as penile discharge. For example, gonorrhoea is one of the causes of 'drip'.

These STIs can occur at various sites of your body including the penis. Common symptoms of STI are penile discharge and pain when urinating. STIs are easily treated with antibiotics providing the right antibiotics are used. An important challenge is that some STIs have become resistant to the antibiotics that are commonly used. This means that the antibiotics that we normally give may not work and that symptoms may not resolve following treatment. This study aims to determine the occurrence of such resistant infections and to identify which antibiotics can best be used to treat discharge in men.

3. Participant selection

You are invited to take part in this study, because you have visited the clinic today to receive treatment for penile or anal discharge that did not resolve after previous treatment. This means that you were recently treated for the same symptoms and that these either remained present or have recurred.

4. Study procedures

If you agree to participate, we will complete a short questionnaire with you that includes questions about who you are, sexual behaviour and medical history including HIV status. We realize that some of these questions are very personal in nature and you must feel free to decline answering any question that you are not comfortable with. For us it is better if you deny answering a question than if you make up the answer. We understand very well that discussing sexual behaviour is a private matter and we will do everything possible to handle your answers in the most discrete manner.

Following these questions, the healthcare worker will perform a physical examination including inspection of the penis and testicles; this is routine clinical care. The healthcare worker will request you to provide a first-void urine specimen for STI testing in case of penile discharge or collect an anal swab in case of anal discharge. Also, a small volume of discharge will be obtained for microscopy. Collection of these specimens is not painful. After collection of samples you are provided with the routine clinical treatment for penile or anal discharge.

The samples will be tested at the Department of Medical Microbiology laboratories of the University of Pretoria. At the laboratory, these specimens are tested for presence of various infections that may cause your symptoms such as *Neisseria gonorrhoeae, Chlamydia trachomatis*, *Trichomonas vaginalis*, *Mycoplasma genitalium*. Also, we will test whether the antibiotics that you were given upon recruitment will work in case of *Neisseria gonorrhoeae* infection or which antibiotics you should receive to cure the infection. Also, we will assess *Mycoplasma genitalium* for potential resistance. In order to better understand the occurrence and spread of drug-resistant infections, we

will conduct additional laboratory analysis of bacteria causing STIs ('molecular typing') of the STIs; this will not have impact on your treatment.

The study clinician will give you a telephone call to discuss the results and if you have an antibiotic resistant infection, we will ask you to visit the clinic again for further treatment.

5. Risks

The questionnaire includes topics such as sexual behaviour and relationships. These questions may be perceived as too explicit or embarrassing to answer. We understand this and will not put any pressure on you to answer questions if you do not feel comfortable about. Should you experience distress following one of the questions, an experienced counsellor is on-site to assist you. Production of urine specimens is considered a safe procedure, whereas obtaining a small sample of penile discharge for microscopy and anal swab for microbiological testing in case of anal discharge, are not painful but may provide a little discomfort. However, these procedures are common practice in other countries where these tests are performed as routine. We commit to maximum effort to provide the most possible private space for collection of samples.

6. Benefits

If you decide to participate in this study, you will be examined by a healthcare worker and additional microscopy and laboratory tests are conducted. This means that information is gathered about your condition that would normally not have been available, especially with regards to the cause of your symptoms and potential drug-resistant infections. Such information allows for possibly better treatment of your condition and certainly a better explanation of what is causing your symptoms and how you got these. This may improve your physical and sexual health. In the broader perspective, the results generated by this study are used to improve clinical management of other people with similar symptoms in the community.

7. Reimbursements

You will not be provided any incentive to take part in the research. Participation is entirely on a voluntary basis.

8. Confidentiality

The information obtained from this research study will be kept confidential. **We will not share any information about you to anyone outside of the research team**. If you decide to participate, you are allocated a study number for identification purposes. Data are collected on separate forms that

have this study number for identification, but never your personal details. As such, the information recorded can never be directly linked to you. We guarantee that the information is not discussed or shared with other people. All information will be kept strictly confidential at any time and will be securely stored at the University of Pretoria for 15 years as per the institutional data retention policy. No other person than the study team has access to this information.

9. Right to refuse or withdraw

You do not have to take part in this research study if you do not wish to do so. If you decide not to participate in this study, you will still receive standard treatment as anyone else. You can decide to withdraw any time after the decision to participate in this study; in that case, we will destroy the information collected from you.

10. Contact information

If you have any questions, you can contact:

- Mr LD Maduna 012 3192250
- Prof RPH Peters: 011 5815000
- Dr MM Kock 012 3192325

Contact details of the University of Pretoria Faculty of Health Science Research Ethics Committee: - 012 356 3084

11. Ethical approval

This Protocol was submitted to the Faculty of Health Sciences Research Ethics Committee, University of Pretoria, telephone numbers 012 356 3084 / 012 356 3085 and written approval has been granted by that committee. The study has been structured in accordance with the Declaration of Helsinki (last update: October 2013), which deals with the recommendations guiding doctors in biomedical research involving human/subjects. A copy of the Declaration may be obtained from the investigator should you wish to review it.

You can ask me any more questions about any part of the research study.
Do you have any questions?

Part II: Certificate of Consent

I have read this informed consent form and that I understand the above information before signing this form. The content and the meaning of this information sheet have been explained to me. I had the opportunity to ask questions regarding this informed consent form and the research study and any questions I have asked have been answered to my satisfaction. I consent voluntarily to be a participant in this study and understand that I will be free to withdraw from the study at any time for any reason.

I have received a copy of this informed consent agreement

Name of Participant _____

Signature of Participant _____

Date _____

Day/month/year

Will you be willing to be contacted for future studies? ☐ **Yes** ☐ **No**

Statement by the researcher/person taking consent

I have accurately read out the information sheet to the potential participant and to the best of my ability made sure that the participant understands the information in the written form.

I confirm that the participant was given an opportunity to ask questions about the study and all the questions asked by the participant have been answered correctly and to the best of my ability. I confirm that the individual has not been coerced into giving consent and that the consent has been given freely and voluntarily.

Name of researcher/person taking the consent _____

Signature of researcher/person taking the consent _____

Date: _____

Day/month/year

Confirmation by witness who is present during the consenting procedure if the patient cannot read or write

Name of Witness _____

Signature of Witness _____

Date _____

Day/month/year

MSM PARTICIPANT'S INFORMATION LEAFLET & INFORMED CONSENT FORM

Dear Mr. _____

Date of consent procedure: ……/……/……

This is an informed consent form for men who visit this healthcare facility to consult with the health care worker. We would appreciate a moment of your time and like to invite you to participate in a research project titled: "**Molecular epidemiology and antimicrobial resistance of *Neisseria gonorrhoeae* in men at risk in Gauteng, South Africa**".

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1. Introduction

You are invited to be part of a research project that we are conducting at this healthcare facility to support the treatment of sexually transmitted infections (STIs) in men. Penile discharge is a common symptom that may suggest presence of an STI, but can also be caused other infections. We are specifically looking for men consulting for STI symptoms (e.g. penile or anal discharge) or men without symptoms but would like to know if they have an STI. This information leaflet serves to inform you about the project and to help you to decide if you would like to participate. Before you decide on whether or not you participate, I would like to explain to you why this study is important, why it may be relevant to you, and what participation involves. Before you agree to take part in this study, you should fully understand what is involved. If you have any questions, do not hesitate to ask the investigator. If you agree to participate, you will be asked to sign a consent form.

2. The aim of the study

Sexually transmitted infections (STIs) are infections that are transmitted through sexual contact and may cause symptoms such as penile discharge or anal discharge. For example, gonorrhoea is one of

the causes of 'drip'. These STIs can occur at various sites of your body including the penis. Common symptoms of STI are penile discharge and pain when urinating. Some STIs can also be present without any obvious signs and individuals with such asymptomatic infection are likely to spread these STIs to their sexual partner as they are both not aware of it. STIs are easily treated with antibiotics providing the right antibiotics are used. An important challenge is that some STIs have become resistant to the antibiotics that are commonly used. This means that the antibiotics that we normally give may not work and that symptoms may not resolve following treatment. This study aims to determine the occurrence of such resistant infections and to identify which antibiotics can best be used to treat discharge in men.

3. Participant selection

You are invited to take part in this study, because you have visited the clinic today to receive treatment for penile or anal discharge or would to know if you have an STI without any obvious signs.

4. Study procedures

If you agree to participate, we will complete a short questionnaire with you that includes questions about who you are, sexual behaviour and medical history including HIV status. We realize that some of these questions are very personal in nature and you must feel free to decline answering any question that you are not comfortable with. For us it is better if you deny answering a question than if you make up the answer. We understand very well that discussing sexual behaviour is a private matter and we will do everything possible to handle your answers in the most discrete manner.

Following these questions, the healthcare worker will request you to provide a first-void urine specimen for STI testing in case of a penile discharge, collect an anal swab in case of anal discharge and oropharyngeal swab in case of a throat infection. Also, a small volume of discharge will be obtained for microscopy. Collection of these specimens is not painful. After collection of samples you are provided with the routine clinical treatment for penile or anal discharge.

The samples will be tested at the Department of Medical Microbiology laboratories of the University of Pretoria. At the laboratory, these specimens are tested for presence of various infections that may cause your symptoms such as *Neisseria gonorrhoeae, Chlamydia trachomatis*, *Trichomonas vaginalis*, *Mycoplasma genitalium*. Also, we will test whether the antibiotics that you were given upon recruitment will work in case of *Neisseria gonorrhoeae* infection or which antibiotics you should receive to cure the infection. Also, we will assess *Mycoplasma genitalium* for potential resistance. In order to better understand the occurrence and spread of drug-resistant infections, we

will conduct additional laboratory analysis of bacteria causing STIs ('molecular typing') of the STIs; this will not have impact on your treatment.

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The questionnaire includes topics such as sexual behaviour and relationships. These questions may be perceived as too explicit or embarrassing to answer. We understand this and will not put any pressure on you to answer questions if you do not feel comfortable about. Should you experience distress following one of the questions, an experienced counsellor is on-site to assist you. Production of urine specimens is considered a safe procedure, whereas obtaining a small sample of penile discharge for microscopy and anal swab for microbiological testing in case of anal discharge, are not painful but may provide a little discomfort. However, these procedures are common practice in other countries where these tests are performed as routine. We commit to maximum effort to provide the most possible private space for collection of samples.

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Do you have any questions?

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I have received a copy of this informed consent agreement

Name of Participant _____

Signature of Participant _____

Date _____

Day/month/year

Will you be willing to be contacted for future studies? ☐ **Yes** ☐ **No**

Statement by the researcher/person taking consent

I have accurately read out the information sheet to the potential participant and to the best of my ability made sure that the participant understands the information in the written form.

I confirm that the participant was given an opportunity to ask questions about the study and all the questions asked by the participant have been answered correctly and to the best of my ability. I confirm that the individual has not been coerced into giving consent and that the consent has been given freely and voluntarily.

Name of researcher/person taking the consent _____

Signature of researcher/person taking the consent _____

Date: _____

Day/month/year

Confirmation by witness who is present during the consenting procedure if the patient cannot read or write

Name of Witness _____

Signature of Witness _____

Date _____

Day/month/year

B. CASE REPORT FORMS

MEN WITH REPEAT STI, PARTICIPANT QUESTIONNAIRE

Molecular epidemiology and antimicrobial resistance of *Neisseria gonorrhoeae* in men at risk in Gauteng, South Africa

Participant ID:_____

Participant ID:

Inclusion date	☐			
Health facility	☐ Chiawelo	☐ Yeoville	☐ Zola	☐ Skinner

SOCIODEMOGRAPHICS

Age (years):				
Nationality	☐ RSA	☐ Other (specify):		
Ethnicity group	☐ Black	☐ White	☐ Coloured	
	☐ Asian	☐ Hispanic	☐ Other	
	☐ If other specify:			
Where do you reside?	☐ Inner City	☐ Suburb	☐ Township	☐ Village
Specify Location				
Highest Level of Education competed	☐ None	☐ Primary	☐ Matric	☐ Tertiary
Source of Income	☐ Formal Employment	☐ Self Employed	☐ Student	☐ Unemployed
Dependent on social grant?	☐ Yes	☐ No		
Sexual identity	☐ Straight	☐ Gay	☐ Bisexual	☐ Undecided
Sexual preference	☐ Women	☐ Men	☐ Both	☐ Decline to answer

Relationship status in the last six months	☐ Stable female	☐ Stable male	☐ Occasional female	☐ Occasional male	☐ both
Most recent sexual event with women	☐ <7 days ago	☐ past 30 days	☐ past 90 days	☐ >6, but <12 months	☐ Not applicable
Number of female sexual partners	☐ In past four weeks:		☐ In last six months:		
Most recent sexual event with men	☐ <7 days ago	☐ past 30 days	☐ past 90 days	☐ >6, but <12 months	☐ Not applicable
Number of male sexual partners	☐ In past four weeks:		☐ In last six months:		

Participant ID:

CLINICAL HISTORY AND PRESENTATION

History of treatment of urethritis/ urethral discharge	Last 5 years:	☐ Yes	☐ No	
	If yes number of times treated?			
	Last 6 months:	☐ Yes	☐ No	
	If yes number of times treated?			
	Time (months) since last treatment:			
History of treatment of anal discharge	Last 5 years:	☐ Yes	☐ No	
	Last 6 months:	☐ Yes	☐ No	
	Time (months) since last treatment:			
Describe last STI treatment	☐ One type of pills	☐ Two types of pills	☐ Injection	
	Name and dosage of drug given			
Did you take your most STI treatment medication	☐ Yes	☐ Did not take		
Place of Last STI treatment	☐ Public	☐ Private	☐ Pharmacy	☐ Traditional healer
	If public specify	☐ Anova H4M	☐Other:	
Traditional STI medication	☐ Yes, less than 6 months	☐ Yes, more than 6 months	☐ None	
After most recent STI treatment, what happened to your symptoms?	☐ Resolved	☐ Resolved and returned	☐ Persistent	
Did you notify any partner after last STI treatment?	☐ Yes, stable	☐ Yes, occasional	☐ Yes, both	☐ No
Did the notified partner go to the clinic for treatment	☐ Yes, I know	☐ Yes, I think so	☐ Don't know	☐ No, didn't go
Did you have sex with the same partner after you were treated for most recent STI?	☐ Yes, same stable partner	☐ Yes, same occasional partner	☐ No, different occasional partners	
Did your relationship status change after last STI treatment?	☐ Yes	☐ No	Specify:	
HIV status at enrolment	☐ Positive	☐ Negative	☐ Unknown	☐ Decline to answer
	If HIV Positive:	☐ On ART	☐ Pre-ART	
If on ART, which regimen are you on?	☐ FDC tablet (Tenofovir + Emtricitabine + Efavirenz)			
	☐ Other, specify:			
Are you currently on PrEP?	☐ Yes	☐ No		

Participant ID:

STABLE PARTNER LAST 6 MONTHS (IF APPLICABLE)

Most stable partner in the last six months?	☐ Yes, female	☐ Yes, male	☐ Yes, both	☐ Not applicable
Do you think your partner has other sex partners besides you? (exclusiveness)	☐ Yes, know so	☐ Yes, think so	☐ don't know	☐ No
Did you have vaginal sex?	☐ Yes	☐ No	☐ Not applicable	
Condom use during most recent vaginal intercourse	☐ Yes	☐ No	☐ Not applicable	
Did you have oral sex? (multiple)	☐ Yes, received blow job	☐ Yes, gave cunnilingus	☐ Yes, gave blowjob	☐ No
Did you have anal sex?	☐ Insertive/Top	☐ Receptive/Bottom	☐ Both/Versatile	☐ No
Condom use during most recent anal intercourse	☐ Yes	☐ No	☐ Not applicable	
Did you have oral-anal contact (rimming)?	☐ Yes	☐ No		
Lubricant use during sexual intercourse	☐ Yes, lubricant	☐ Yes, saliva	☐ Yes, other	☐ None
Alcohol use before recent sex act	☐ Most of the times	☐ Sometimes	☐ Never	
Drugs (e.g dagga, Nyaope, Meth) use before recent sex act	☐ Most of the times	☐ Sometimes	☐ Never	
	If yes, specify type of drug used:			

Participant ID:

OCASSIONAL PARTNER LAST 6 MONTHS (IF APPLICABLE)

Most recent sex partner in the last six months?	☐ Yes female	☐ Yes, male	☐ None	☐ Both
How did you meet your recent sexual partner (s) (multiple answers)	☐ Internet or social media	☐ Bar/Club	☐ Sauna/Bathhouse	
	☐ Sex parties	☐ Other:		
Have you received money or goods for sex?	☐ No	☐ Yes, from woman	☐ Yes, from man	☐ Yes, from both
	If yes, do you consider yourself a sex worker?		☐ Yes	☐ No
Have you paid for sex in the last six months?	☐ No	☐ Yes, woman	☐ Yes, man	☐ Yes, both
	If yes, was this person a sex worker?		☐ Yes	☐ No
Did you know your sex partner's name?	☐ Yes	☐ No	☐ Decline to answer	
Did you have vaginal sex?	☐ Yes	☐ No	☐ Not applicable	
Condom use during most recent vaginal intercourse	☐ Yes	☐ No	☐ Not applicable	
Did you have oral sex? (multiple)	☐ Yes, received blow job	☐ Yes, gave cunnilingus	☐ Yes, gave blowjob	☐ No
Did you have anal sex?	☐ Insertive/Top	☐ Receptive or Bottom	☐ Both/Versatile	☐ No
Condom use during most recent anal intercourse	☐ Yes	☐ No	☐ Not applicable	
Did you have oral-anal contact (rimming)?	☐ Yes	☐ No		
Lubricant use during sexual intercourse	☐ Yes, saliva	☐ Yes, lubricant	☐ Yes, other	☐ None
Sex with >1 partner at the same time in last six months	☐ Once	☐ Occasionally	☐ Never	
Alcohol use before recent sex act	☐ Most of the times	☐ Sometimes	☐ Never	
Drugs (e.g Dagga, Nyaope, Meth) use before recent sex act	☐ Most of the times	☐ Sometimes	☐ Never	
	If yes, specify type of drug used:			

Participant ID:

CLINICAL HISTORY AND PRESENTATION

Current STI symptoms at present (multiple answers)	Genital symptoms	☐ penile discharge	☐ Dysuria
		☐ urethral itch	☐ Testicular pain
		☐ Inguinal swelling	☐ Inguinal pain
	Anal symptoms:	☐ Anal discharge	☐ Anal itch
		☐ Rectal bleeding	☐ Tenesmus
	Oral symptoms:	☐ painful throat and difficulty swallowing ☐ sores in the mouth ☐ Whitish/yellowish discharge	
	Other symptoms:		

PHYSICAL EXAMINATION

Urethral discharge observed?	☐ Yes, on penis	☐ Yes, underwear only	☐ No
	If discharge	☐ Colour:	
	Any abnormalities		
Inguinal lymphadenopathy	☐ Present	☐ Absent	
Testicular abnormalities	☐ Pain	☐ Swelling	☐ None
	Specify:		
Anal discharge observed?	☐ Yes	☐ No	
	Any anal abnormalities?		
Enlarged tonsils with signs of inflammation?	☐ Yes	☐ No	
Inguinal lymphadenopathy	☐ present	☐ absent	
Any other abnormalities during examination?			

Participant ID:

CLINICAL MANAGEMENT AT INCLUSION

Treatment given	Azithromycin, Oral	☐ 1g, single dose	☐ 2g, single dose
	Ceftriaxone, IM	☐ 250mg, single dose	☐ 1g, single dose
	Metronidazole, Oral	☐ 2g, single dose	☐ 400g x 7 days
	Gentamycin, IM	☐ 240mg, single dose	
	Doxycline, Oral	☐ 100mg X 7 days	
	Other treatment		
Partner notification	☐ Will notify stable partner	☐ Will notify occasional partner	☐ Does not know partners
Partner notification slip issued	☐ Yes	☐ No	

LABORATORY TESTING

First void urine sample collected	☐ Yes
Discharge culture prepared	☐ Yes
Other tests	

Participant ID:

PARTICIPANT FOLLOW UP

PCR results	☐ NG positive	☐ CT positive	☐ TV positive	☐ MG positive
Culture	☐ NG positive	☐ NG negative	☐ Neisseria sp	
Culture positive antibiogram	Ceftriaxone (CRO)	☐ S	☐ I	☐ R
	Azithromycin (AZM)	☐ S	☐ I	☐ R
	Cefixime (CFM)	☐ S	☐ I	☐ R
	Spectinomycin (SPT)	☐ S	☐ I	☐ R
	Ciprofloxacin (CIP)	☐ S	☐ I	☐ R
	Penicillin (PEN)	☐ S	☐ I	☐ R
	Tetracycline (TET)	☐ S	☐ I	☐ R
Interpretation of results				
Follow up	Patient called to discuss results	☐ Yes		
	Patient adequately treated	☐ Yes		
	Follow up visit explained	☐ Yes		
Date follow -up scheduled				
Did the patient show up for the follow up treatment	☐ Yes	☐ No		
	Details of management			

MSM PARTICIPANT QUESTIONNAIRE

Molecular epidemiology and antimicrobial resistance of *Neisseria gonorrhoeae* in men at risk in Gauteng, South Africa

Participant ID:_____

Participant ID:

Inclusion date			
Health facility	☐ Chiawelo	☐ Yeoville	☐ Zola
How did you hear/ find out about the study	☐ Through a friend	☐ Have symptoms (e.g discharge, burning sensation)	
	☐ Through a clinic poster	☐ Get free STI testing	
	☐ High-risk behaviour	☐ Visiting clinic for other condition	
	☐ Community outreach	☐ Other, specify:	

SOCIODEMOGRAPHICS

Age (years):				
Nationality	☐ RSA	☐ Other (specify):		
Ethnicity group	☐ Black	☐ White	☐ Coloured	
	☐ Asian	☐ Hispanic	☐ Other	
	☐ If other specify:			
Where do you reside?	☐ Inner City	☐ Suburb	☐ Township	☐ Village
Specify Location				
Highest Level of Education competed	☐ None	☐ Primary	☐ Matric	☐ Tertiary
Source of Income	☐ Formal Employment	☐ Self Employed	☐ Student	☐ Unemployed
Dependent on social grant?	☐ Yes	☐ No		
Sexual identity	☐ Gay	☐ Straight	☐ Bisexual	☐ Undecided
Sexual preference	☐ Men	☐ Women	☐ Both	☐ Decline to answer
Relationship status in the last six months	☐ Stable male	☐ Stable female	☐ Occasional male	☐ Occasional female	☐ Both
Most recent sexual event with men	☐ <7 days ago	☐ past 30 days	☐ past 90 days	☐ >6, but <12 months
Number of male sexual partners	☐ In past four weeks:	☐ In last 6 months:		
Most recent sexual event with women	☐ <7 days ago	☐ past 30 days	☐ past 90 days	☐ >6, but <12 months	☐ Not applicable
Number of female sexual partners	☐ In past four weeks:	☐ In last 6 months:		

Participant ID:

SEXUAL BEHAVIOUR-STABLE PARTNER IN THE LAST 6 MONTHS

Most stable partner in the last six months?	☐ Yes, male	☐ Yes, female	☐ Yes, both	☐ Not applicable
Do you think your partner has other sex partners besides you? (exclusiveness)	☐ Yes, know so	☐ Yes, think so	☐ don't know	☐ No
Did you have anal sex?	☐ Yes, gave	☐ Yes, received	☐ Yes, both	☐ No
Condom use during most recent anal intercourse	☐ Yes, partner used as top	☐ Yes, I used as top	☐ No	
Lubricant use during sexual intercourse	☐ Yes, lubricant	☐ Yes, saliva	☐ Yes, other	☐ None
Did you have oral sex? (blow job)	☐ Yes, gave	☐ Yes, received	☐ Yes, both	☐ No
Did you have oral-anal contact (rimming)?	☐ Yes	☐ No		
Did you have vaginal sex?	☐ Yes	☐ No	☐ Not applicable	
Condom use during most recent vaginal intercourse	☐ Yes	☐ No	☐ Not applicable	
Alcohol use before recent sex act	☐ Most of the times	☐ Sometimes	☐ Never	
Drugs (e.g dagga, Nyaope, Meth) use before recent sex act	☐ Most of the times	☐ Sometimes	☐ Never	
	If yes, specify type of drug used:			
Any comments?				

Participant ID:

SEXUAL BEHAVIOUR-OCASSIONAL PARTNER (S) IN THE LAST SIX MONTHS

Most recent sex partner in last 6 months	☐ Yes, male	☐ Yes, female	Yes, both	
How did you meet your recent sexual partner (s) (multiple answers)	☐ Internet or social media	☐ Bar/Club	☐ Sauna/Bathhouse	
	☐ Sex parties	☐ Other:		
Did you know your last sex partner's name?	☐ Yes	☐ No	☐ Decline to answer	
Have you paid for sex in the last six months?	☐ No	☐ Yes, woman	☐ Yes, man	☐ Yes, both
	If yes, was this person a sex worker?	☐ Yes	☐ No	
Have you received money or goods for sex?	☐ No	☐ Yes, from woman	☐ Yes, from man	☐ Yes, from both
	If yes, do you consider yourself a sex worker?	☐ Yes	☐ No	
Did you have anal sex?	☐ Yes, gave	☐ Yes, received	☐ Yes, both	☐ No
Condom use during most recent anal intercourse	☐ Yes, partner used as top	☐ Yes, I used as top	☐ No	
Did you have oral sex? (blow job)	☐ Yes, gave	☐ Yes, received	☐ Yes, both	☐ No
Did you have oral-anal contact (rimming)?	☐ Yes	☐ No		
Lubricant use during sexual intercourse	☐ Yes, saliva	☐ Yes, lubricant	☐ Yes, other	☐ None
Sex with >1 partner at the same time in last six months	☐ Once	☐ Occasionally	☐ Never	
Did you have vaginal sex?	☐ Yes	☐ No	☐ Not applicable	
Condom use during most recent vaginal intercourse	☐ Yes	☐ No	☐ Not applicable	
Alcohol use before recent sex act	☐ Most of the times	☐ Sometimes	☐ Never	
Drugs (e.g dagga, Nyaope, Meth) use before recent sex act	☐ Most of the times	☐ Sometimes	☐ Never	
	If yes, specify type of drug used:			
Any comments?				

Participant ID:

CLINICAL HISTORY AND PRESENTATION

History of treatment of urethritis/urethral discharge	Last 5 years:	☐ Yes	☐ No	
	If yes number of times treated?			
	Last 6 months:	☐ Yes	☐ No	
	If yes number of times treated?			
	Time (months) since last treatment			
History of treatment of anal discharge	Last 5 years:	☐ Yes	☐ No	
	If yes number of times treated?			
	Last 6 months:	☐ Yes	☐ No	
	If yes, no of times			
	Time (months) since last treatment			
Number of times treated for any STI in the last 6 months				
Place of Last STI treatment	☐ Public	☐ Private	☐ Pharmacy	
Traditional STI medication	☐ Yes, less than 6 months	☐ Yes, more than 6 months	☐ None	
HIV status at enrolment	☐ Positive	☐ Negative	☐ Unknown	☐ Decline to answer
	If HIV Positive:	☐ On ART	☐ Pre-ART	
If on ART, which regimen are you on?	☐ FDC tablet (Tenofovir + Emtricitabine + Efavirenz)			
	☐ Other, specify:			
Are you currently on PrEP?	☐ Yes	☐ No		
STI symptoms present?	☐ Yes	☐ No		
Current STI symptoms at present (multiple answers)	Genital symptoms	☐ Penile discharge	☐ Dysuria	
		☐ Urethral itch	☐ Testicular pain	
		☐ Inguinal swelling	☐ Inguinal pain	
	Anal symptoms:	☐ Anal discharge	☐ Anal itch	
		☐ Rectal bleeding	☐ Tenesmus	
	Oral symptoms:	☐ painful throat and difficulty swallowing ☐ sores in the mouth ☐ Whitish/yellowish discharge		
	Other symptoms:			

Participant ID:

LABORATORY TESTING

First void urine sample collected	☐ Yes
Rectal swab collected	☐ Yes, HCW collected
Pharyngeal swab collected	☐ Yes
Other tests	

PHYSICAL EXAMINATION

Urethral discharge observed?	☐ Yes, on penis	☐ Yes, underwear only	☐ No
	If discharge	☐ Colour:	☐ Smell:
	Any abnormalities		
Inguinal lymphadenopathy	☐ present	☐ absent	
Testicular abnormalities	☐ Pain	☐ Swelling	☐ None
	Specify:		
Anal discharge observed?	☐ Yes	☐ No	
	Any anal abnormalities?		
Enlarged tonsils with signs of inflammation?	☐ Yes	☐ No	
Any other abnormalities during examination?			

Participant ID:

CLINICAL MANAGEMENT AT INCLUSION

Treatment given	Azithromycin, Oral	☐ 1g, single dose	☐ 2g, single dose
	Ceftriaxone, IM	☐ 250mg, single dose	☐ 1g, single dose
	Metronidazole, Oral	☐ 2g, single dose	☐ 400g x 7 days
	Gentamycin, IM	☐ 240mg, single dose	
	Doxycline, Oral	☐ 100mg X 7 days	
	Other treatment		
Partner notification	☐ Will notify stable partner	☐ Will notify occasional partner	☐ Does not know partners
Partner notification slip issued	☐ Yes	☐ No	

Participant ID:

PARTICIPANT FOLLOW UP

Urine PCR	☐ NG positive	☐ CT positive	☐ TV positive	☐ MG positive
NG culture	☐ NG positive	☐ NG negative	☐ Neisseria sp	
Culture	Ceftriaxone (CRO)	☐ S	☐ I	☐ R
	Azithromycin (AZM)	☐ S	☐ I	☐ R
	Cefixime (CFM)	☐ S	☐ I	☐ R
	Spectinomycin (SPT)	☐ S	☐ I	☐ R
	Ciprofloxacin (CIP)	☐ S	☐ I	☐ R
	Penicillin (PEN)	☐ S	☐ I	☐ R
	Tetracycline (TET)	☐ S	☐ I	☐ R
Rectal PCR	☐ NG positive	☐ CT positive	☐ TV positive	☐ MG positive
NG culture	☐ NG positive	☐ NG negative	☐ Neisseria sp	
Culture positive antibiogram	Ceftriaxone (CRO)	☐ S	☐ I	☐ R
	Azithromycin (AZM)	☐ S	☐ I	☐ R
	Cefixime (CFM)	☐ S	☐ I	☐ R
	Spectinomycin (SPT)	☐ S	☐ I	☐ R
	Ciprofloxacin (CIP)	☐ S	☐ I	☐ R
	Penicillin (PEN)	☐ S	☐ I	☐ R
	Tetracycline (TET)	☐ S	☐ I	☐ R

Participant ID:

Throat PCR	☐ NG positive	☐ CT positive	☐ TV positive	☐ MG positive
NG culture	☐ NG positive	☐ NG negative	☐ Neisseria sp	
Culture positive antibiogram	Ceftriaxone (CRO)	☐ S	☐ I	☐ R
	Azithromycin (AZM)	☐ S	☐ I	☐ R
	Cefixime (CFM)	☐ S	☐ I	☐ R
	Spectinomycin (SPT)	☐ S	☐ I	☐ R
	Ciprofloxacin (CIP)	☐ S	☐ I	☐ R
	Penicillin (PEN)	☐ S	☐ I	☐ R
	Tetracycline (TET)	☐ S	☐ I	☐ R
Interpretation of results				

Participant ID:

PARTICIPANT FOLLOW UP

Follow up	Patient called to discuss results	☐ Yes	
	Patient adequately treated?	☐ Yes	☐ No, follow up required
	Follow up visit explained?	☐ Yes	☐ No
Date follow -up scheduled			
Did the patient show up for the follow up treatment	☐ Yes	☐ No	
	Details of management		

APPENDIX C Experimental procedures

PART I

Protocol for extraction of Deoxyribonucleic acid (DNA)

Urine, genital swabs and culture were processed for DNA extraction using the High Pure PCR Template Preparation Kit (Roche diagnostics, Germany) with a modification of Step 1 (De Waaij *et al.*, 2015).

Step 1

- To a nuclease-free 1.5 mL microcentrifuge tube (Eppendorf, Germany):
- Add 200 µL of sample material.
- Add 5 µl of extraction positive control
- Add 200 µL Binding Buffer.
- Add 40 µL Proteinase K (reconstituted).
- Mix immediately and incubate at + 70°C (AccuBlock digital dry bath, Labnet, Edison, USA) for 10 min.

Step 2

- Add 100 µL Isopropanol (Merck, Germany) and mix well.

Step 3

- Insert one High Pure Filter Tube into one Collection Tube.
- Pipet the sample into the upper buffer reservoir of the Filter Tube.
- Insert the entire High Pure Filter Tube assembly into a standard table-top centrifuge.
- Centrifuge (Eppendorf, Hamburg, Germany) for 1 min at 8,000 × g.

Step 4

- After centrifugation:

- Remove the Filter Tube from the Collection Tube; discard the flowthrough and the Collection Tube.
- Combine the Filter Tube with a new Collection Tube.
- Add 500 µL Inhibitor Removal Buffer to the upper reservoir of the Filter Tube.
- Centrifuge (Eppendorf, Hamburg, Germany) 1 min at 8,000 × g.

Step 5

- Remove the Filter Tube from the Collection Tube; discard the flowthrough and the Collection Tube.
- Combine the Filter Tube with a new Collection Tube.
- Add 500 µL Wash Buffer to the upper reservoir of the Filter Tube.
- Centrifuge (Eppendorf, Hamburg, Germany) 1 min at 8,000 × g and discard the flowthrough.

Step 6

- Remove the Filter Tube from the Collection Tube; discard the flowthrough and the Collection Tube.
- Combine the Filter Tube with a new Collection Tube.
- Add 500 µL Wash Buffer to the upper reservoir of the Filter Tube.
- Centrifuge (Eppendorf, Hamburg, Germany) 1 min at 8,000 × g and discard the flow through.

Step 7

- After discarding the flowthrough:
- Centrifuge (Eppendorf, Hamburg, Germany) the entire High Pure assembly for an additional 10 s at 16, 000 x g.
- Discard the Collection Tube.

Step 8

- To elute the DNA:
- Insert the Filter Tube into a clean, sterile 1.5 ml microcentrifuge tube.

- Add 200 µL prewarmed Elution Buffer to the upper reservoir of the Filter Tube.
- Centrifuge (Eppendorf, Hamburg, Germany) the tube assembly for 1 min at 8,000 × g.
- Store DNA at -20°C (Thermo Scientific, USA) for later analysis

Protocol for Gram-stained smear (Hucker, 1921; Maszenan *et al.*, 2000)

Hucker's modification of Gram stain for examination of smears

- Prepare a smear and heat gently on a Bunsen burner to fix
- Flood the slide with 0.5% crystal violet (Diagnostic Media Products, NHLS, South Africa) and leave for 30sec
- Tilt the slide, and rinse slide gently with water
- Flood on sufficient (1%) Lugol's iodine (Diagnostic Media Products, NHLS, South Africa) to rinse off excess water, cover with fresh iodine and allow to remain for 30sec
- Tilt the slide and wash off the iodine with water
- Decolourise with 95 - 100% ethanol (Diagnostic Media Products, NHLS, South Africa) until colour ceases to run out of the smear
- Rinse with water
- Flood the slide 0.1% counterstain safranin (Diagnostic Media Products, NHLS, South Africa) and leave to act for about 30sec to 1min

Part II:

Protocol for culture-free *Neisseria gonorrhoeae* multiantigen sequencing typing (NG-MAST) (Van Der Veer *et al.*, 2018)

I. Bigdye Sequencing NG-MAST

a) Primers:

Primers (0,05 µM scale Purified) were ordered at Sigma-Aldrich. Lyophilised primers were dissolved and diluted in NASBA water (bioMérieux, France). Primers were diluted to 100 pmol/µl stock solution.

b) Primers for sequencing *porB* and *tbpB* genes

	Primer	Sequence (5'-3')
porB	*porB*_F	CAA GAA GAC CTC GGC AA
	*porB*_R	CCG ACA ACC ACT TGG T
tbpB	*tbpB*_F	CGT TGT CGG CAG CGC GAA AAC
	*tbpB*_R	TTC ATC GGT GCG CTC GCC TTG

c) Primer work solution:

For sequencing, primer work solutions of 1 pmol/µl are needed.

d) Reaction mixture:

For this assay, 4 reactions per sample (one reaction per primer) were carried out in a total volume of 10 µL per reaction, containing:

H2O	3.5	µL
BDT buffer 1.1	1.5	µL
Primer (1 uM)	2	µL
Bigdye Terminator 1.1 enzyme mix	1	µL
Purified PCR product	2	µL

e) Bigdye Terminator (BDT) 1.1 program (PCR):

The amplification is conducted as follows:

	temp ºC	**time**
Step1.	96	1 min
Step2.	96	10 sec
	55 (*porB*) / 65 (*tbpB*)	10 sec
	60	3 min 25x
Step3.	4	∞

f) Sequencing:

Sequencing of the samples was performed at Maastricht University Medical Centre using the ABI 3730 (Applied Biosystems)

II. Protocol for PCR-product purification with poly-ethylene glycol

This assay was performed to remove primers before sequencing

a) Procedure:

- Prepare 20% Poly-ethylene glycol (PEG) (Merck, Germany) 2.5M NaCl (Merck, Germany) by adding 7.3 g NaCl (Merck, Germany) and 10g PEG (Merck, Germany) in a 50 ml falcon tube. Dissolve and incubate a 37°C.
- Add 1 volume of PEG solution in a 2 ml microcentrifuge tube (Eppendorf, Hamburg, Germany). Add 50 µL for a 50 µL PCR reaction
- Add PCR reaction mixture and mix by pipetting up and down
- Incubate at 37°C for 15 min followed by centrifuging (Eppendorf, Hamburg, Germany) 15 min. at 15000 x g
- Remove and discard supernatant with a P 200 pipet and add 125 µL cold 80% EtOH
- Centrifuge for 2 min at 15, 000 x g
- Remove and discard supernatant with a P 200 pipet
- Remove and discard supernatant again with a P 200 pipet and add 125 µL cold 80% Ethanol (Merck, Germany).
- Dry pellet and dissolve PCR product in 25 µL NASBA water (bioMérieux, France).
- Check purification by running on agarose gel using 4 µL sample

PART III

Protocol for whole genome sequence (WGS) *Neisseria gonorrhoeae* on the Miseq instrument (Illumina Inc, Madison, USA). Adapted from Sabine Chapelle, Mandy Andriessen, Basil Britto Xavier and Christine Lammens

Principle

Neisseria gonorrhoeae DNA was extracted from the 24-hour culture and the quality of the DNA was checked followed preparation of WGS libraries using NexteraXT library preparation kit (Illumina, the Netherlands)

Step 1: Quality check

A. Protocol for DNA clean and concentration for *Neisseria gonorrhoeae*

1. In a 1.5 ml microcentrifuge tube (Eppendorf, Germany) add 15 µL *N. gonorrhoeae* DNA + 40 µL of Chip DNA binding buffer. Mix briefly by vortexing for 10s.
2. Transfer the mixture to a Zymo-Spin Column (Zymo research, USA) in a Collection Tube
3. Centrifuge at 10000 x g for 30sec. Discard the flow through
4. Add 200 µL DNA Wash Buffer to the column. Centrifuge at 10000 x g for 1min. Repeat the wash step
5. Transfer the column to a 1.5ml microcentrifuge tube (Eppendorf, Germany) and add 15 µL DNA Elution buffer directly to the column matrix. Incubate at room temperature (27°C ± 3°C) for 1min. Centrifuge (Eppendorf, Hamburg, Germany) at 10000 x g for 30sec to elute the DNA.

B. Adjustment of DNA concentration - Qubit® dsDNA HS Assay Kit

Protocol:

1. Before starting the experiments, take out the Qubit dsDNA HS Assay Kit (ThermoFisher Scientific, USA) out of the fridge, let it calibrate to room temperature (27°C ± 3°C) in a dark place
2. Make 1/20 dilution for each purified DNA sample and 1/40 for each non-purified DNA sample with Nuclease Free water (Qiagen, United Kingdom)

3. Set up the number of 0.5 mL Qubit assay tubes needed for standards and samples.
4. The Qubit dsDNA HS Assay Kit (Qiagen, United Kingdom) requires 2 standards.
5. Prepare a Qubit working solution by diluting Qubit dsDNA HS reagent 1:200 in Qubit dsDNA HS buffer. For each sample and standard 200 µL working solution is needed. Mix by pipetting up and down 10 times
6. Load 190 µL of Qubit working solution into each of the tubes used for standards.
7. Add 10 µL of each Qubit standard to the appropriate tube and mix by vortexing at medium speed for 3sec and being careful not to create bubbles.
8. Load 198 µL Qubit working solution into individual assay tubes.
9. Add 2 µL of each sample to the corresponding assay tube. Mix by vortexing (medium speed) 3 sec.
10. Allow all tubes to incubate at room temperature (27°C ± 3°C) for 2min.
11. Plug in the QubitFluorometer
    a. Execute following steps:
    b. Quant-iT dsDNA HS
    c. Run new calibration
    d. Insert Standard 1
    e. Reading complete, insert Standard 2
    f. Reading complete, insert next sample
    g. Fluorometer is ready to use.
    h. Insert the first sample tube, close the lid and press “Read”.
    i. The concentration is displayed, this is the concentration of the diluted 2 µL sample in 198 µL of Qubit Working solution. Press “Calculate Sample Concentration” followed by “2 µL”. The initial concentration is then displayed. Note down.
12. Repeat sample readings until all samples have been read, note down the initial concentrations
13. Dilute the purified DNA’s 1:20, measure with Qubit, and dilute the DNA’s again until the correct concentration.
14. Store the adjusted DNA in the -20°C freezer.

Step 2: Library preparation using NexteraXT DNA sample preparation kit (Illumina Inc, Madison, USA)

C. Tagmentation of input DNA

Preparations before the experiments:

1. Remove Amplicon Tagment Mix (ATM), Tagment DNA Buffer (TD) from freezer (-20°C) (Thermo Scientific, USA) and thaw on ice.
2. Keep the Neutralize Tagment Buffer (NT) at room temperature (27°C ± 3°C) to ensure there is no precipitate. If there is precipitate, vortex until all particulates are resuspended.
3. After thawing, ensure all reagents are adequately mixed by gently inverting the tubes 5 times, followed by a brief spin in a micro centrifuge.
4. Switch on the QuantStudio 5 (ThermoFisher Scientific, USA) thermocycler

Protocol:

1. Label the 96-well PCR plate with NTA (Nextera XT Tagment Amplicon Plate).
2. Add 10 µL of TD Buffer according to the sample sheet. Change tips between wells.
3. Mix the input DNA by tapping the tube and spin down briefly
4. Add 4 µL of input DNA to each sample well of the NTA plate according to the sample sheet.
5. Adjust the volume to 15 µL by adding 1 µL of RNA free water (ThermoFisher Scientific, USA)
6. Add 5 µL of ATM to the wells containing input DNA and TD Buffer. Gently mix the sample by pipetting up and down 5 times. Change tips between samples.
7. Cover the NTA plate with a microseal.
8. Centrifuge at 280xg at RT for 1 min.
9. Place the NTA plate in the QuantStudio 5 (ThermoFisher Scientific, USA) and run the Illumina tagmentation program (55°C for 5 min, cool down to 10°C).
10. After 7 min the thermocycler program is finished, then proceed immediately to next step.
11. Carefully remove the microseal and add 5 µL of NT Buffer to each well of the NTA plate. Gently mix the sample by pipetting up and down 5 times. Change tips between samples.
12. Cover the NTA plate with a new microseal
13. Centrifuge at 280 x g at 20°C for 1 min
14. Place the NTA plate at room temperature (27°C ± 3°C) for 5min

D. PCR Amplification

Preparations before the experiments:

1. Remove Nextera PCR MasterMix (NPM) and the index primers from -20°C freezer and thaw on a bench at room temperature. Allow to thaw for 20 min
2. After all reagents are completely thawed, (27°C ± 3°C) gently invert each tube 5 times to mix and briefly centrifuge the tubes in a micro centrifuge. Place the index tubes in 1.5 mL tubes to centrifuge. (screw of the cap)

Protocol:

1. Add 15 µL of NPM to each well of the NTA plate containing sample. Change tips between samples.
2. Add 5 µL of index 2 primers to the respective wells of the NTA plate, according to the sample sheet. Change tips between samples. To avoid cross contamination, discard the original white caps and apply a new white cap.
3. Add 5 µL of index 1 primers to the respective wells of the NTA plate, according to the sample sheet. Gently mix the sample by pipetting up and down 5 times. Change tips between samples. To avoid cross contamination, discard the original orange caps and apply a new orange cap.
4. Cover the plate with new microseal film
5. Centrifuge at 280 x g at room temperature (27°C ± 3°C) for 1min
6. Perform PCR using the following program on a QuantStudio 5 (ThermoFisher Scientific, USA) thermal cycler:
    a. 70°C for 3 min
    b. 95°C for 30 sec
    c. 12 cycles of
        i. 95°C for 10 sec
        ii. 55°C for 30 sec
        iii. 72°C for 30 sec
    d. 72°C for 5 min
    e. Hold at 10°C

E. PCR clean-up

Preparations before the experiments:

1. Bring AMPure XP beads to room temperature (27°C ± 3°C)
2. Prepare fresh 80% ethanol
3. Remove Resuspension buffer (RSB) from freezer, thaw on ice

Protocol

1. Centrifuge the NTA plate at 280xg for 1min to collect condensation.
2. Label a new 96-well plate with CAA (Clean Amplified Plate)
3. Using a pipette set to 50 µL, transfer the PCR product from the NTA plate to the CAA plate. Change tips between samples.
4. Vortex the AMPure XP beads for 30sec to ensure that the beads are evenly dispersed. Add an appropriate volume of beads in a 1,5 mL microcentrifuge tube (Eppendorf, Germany).
5. Add 30 µL of AMPure XP beads to each well of the CAA plate. Gently mix the samples by pipetting up and down 10 times. Change tips between samples.
6. Incubate at room temperature (27°C ± 3°C) without shaking for 5 min, cover the plate from air with seal
7. Place the plate on a magnetic stand until the supernatant has cleared.
8. With the CAA plate on the magnetic stand, remove and discard the supernatant. Change tips between samples.
9. With the CAA plate on the magnetic stand, wash the beads with freshly prepared 80% ethanol as follows:
    a. Add 200 µL of freshly prepared 80% ethanol to each sample well by using the multichannel pipette
    b. Incubate leave the plate on the magnetic stand for 30sec.
    c. Carefully remove and discard the supernatant by using the multichannel pipet
10. With the CAA plate on the magnetic stand, wash the beads with freshly prepared 80% ethanol as follows:
    a. Add 200 µL of freshly prepared 80% ethanol to each sample well by using the multichannel pipet
    b. Incubate leave the plate on the magnetic stand for 30 sec.
    c. Carefully remove and discard the supernatant by using the multichannel pipet

11. With the CAA plate still on the magnetic stand, allow the beads to air-dry for 15 min, covered with a Kleenex, under the laminar flow. Visually check whether beads are dry, if not, allow to air-dry further for 5 min.
12. Remove the CAA plate from the magnetic stand. Add 52,5 µL of RSB to each well of the CAA plate. Gently mix the sample by pipetting up and down 10 times. Change tips between samples.
13. Incubate at room temperature (27°C ± 3°C) for 2 min.
14. Place the plate on the magnetic stand until the supernatant has cleared.
15. Label a new 96-well plate CAN (Clean Amplified NTA Plate), or use the lower part of the CAA plate itself if possible
16. Carefully transfer 50 µL of the supernatant from the CAA plate to the CAN plate (or to the bottom of the CAA plate). Change tips between samples to avoid cross contamination

F. Library Normalisation

Preparations before the experiments:

1. This set of reagents contains formamide (Merck, Germany), working under fume hood is required and dispose contents in appropriate container.
2. Remove Library Normalisation Additives 1 (LNA1) from -20°C and bring to room temperature (27°C ± 3°C). Before use, vortex vigorously.
3. Remove Library Normalisation Beads 1 (LNB) and Library Normalisation Wash 1 (LNW1) from 4°C storage and bring to room temperature.
4. Vigorously vortex LNB for at least 1min with intermittent inversion until the beads are well-resuspended and no pellet is found at the bottom of the tube when the tube is inverted.
5. Ensure that LNS1 is at room temperature before use.
6. Prepare 0,1N NaOH (=0,2g/50 mL). Dissolve 0,2g NaOH in 50 mL MilliQ.
7. Prepare a sufficient amount of LNA1-LNB1 suspension by mixing 46 µL LNA1/sample and 8,4 µL LNB1/sample in an microcentrifuge tube.
    a. Note: Pay attention to the LNB1, beads should be resuspended as described above.
    b. Note: When pipetting the beads, visually check the pipet tips. There should not be any excessive drops of beads present. This may influence the normalization.

Protocol

1. Label a new plate LNP (Library Normalization Plate)
2. Carefully transfer 20 µL of the supernatant from the CAN plate to the LNP plate. Change tips between samples to avoid cross-contamination.
3. Add 45 µL of the combined LNA1/LNB1 bead mix to each well of the LNP plate containing libraries. Change tips between samples.
4. (!) When pipetting the beads, visually check the pipet tips. There should not be any excessive drops of beads present. This may influence the normalization.
5. Seal the LNP plate with microseal.
6. Shake the plate on a microplate shaker at 1800 rpm for 30min.
7. Place the plate on a magnetic stand until the supernatant has cleared.
8. With the LNP plate on the magnetic stand, set the pipette at 80 µL and carefully remove the supernatant. Discard the supernatant in an appropriate hazardous waste container.
9. Remove the LNP plate from the magnetic stand and wash the beads with LNW1 as follows
    a. Add 45 µL of LNW1 to each sample well. Change tips between samples.
    b. Seal the LNP plate with microseal
    c. Shake the LNP plate on a microplate shaker at 1800 rpm for 5min.
    d. Centrifuge at 280 x g at 20°C for 30 xsec
    e. Place the plate on the magnetic stand until the supernatant has cleared.
    f. Carefully remove and discard the supernatant in an appropriate hazardous waste container
10. Remove the LNP plate from the magnetic stand and repeat the wash with LNW1 as follows
    a. Add 45 µL of LNW1 to each sample well. Change tips between samples.
    b. Seal the LNP plate with microseal
    c. Shake the LNP plate on a microplate shaker at 1800 rpm for 5 min.
    d. Centrifuge at 280 x g at 20°C for 30 sec
    e. Place the plate on the magnetic stand until the supernatant has cleared.
    f. Carefully remove and discard the supernatant in an appropriate hazardous waste container.
11. Remove the LNP plate from the magnetic stand and add 30 µL of 0.1N NaOH to each well to elute the sample.

12. Seal the LNP plate with microseal
13. Shake the LNP plate on a microplate shaker at 1800 rpm for 5 min.
14. Centrifuge at 280 x g at 20°C for 30 sec

    (!) After 5 min of elution, ensure all samples in the LNP plate are completely resuspended. If the samples are not completely resuspended, gently pipette those samples up and down or lightly tap the plate on the bench to resuspend the beads, then shake for another 5 min at 1800 rpm.
15. Prepare a new 96-PCR plate by labelling it SGP (Storage Plate)
16. Add 30 µL of LNS1 to each well to be used in the SGP plate
17. Place the LNP plate on the magnetic stand until the supernatant appears clear.
18. Transfer 30 µL of the supernatant from the LNP plate to the SGP plate. Change tips between samples.
19. Seal the SGC plate with microseal and then centrifuge at 1000 x g for 1min.
20. Store the plate at -20°C (max 3weeks) wrapped in a plastic bag and labelled properly.

Step 3: MiSeq sequencing run

G. Step XII – Library Pooling for MiSeq Sequencing

Preparations before the experiments:

1. Set a heat block suitable for 1.5 mL microcentrifuge tube (Eppendorf, Germany) to 96°C
2. Thaw Hybridisation buffer (HT1) on ice
3. Remove a MiSeq reagent cartridge from freezer and thaw RT water bath for 90min. Make sure that all reagents are fully thawed.
4. Manually invert cartridge 10X
5. Tap cartridge on hard surface to remove bubbles and dislodge water from cartridge base.
6. Put cartridge on ice or in cold room until beginning run
7. If the SGP plate was frozen, using a P 200 pipette, mix each library to be sequenced by pipetting up and down 5 times. Change tips between samples.
8. Prepare 0.2N NaOH (45 µL distilled water+ 5 µL Stock 1.0N NaOH). Mix by inverting few times. (A fresh dilution of 0.2N NaOH is required for the denaturation process in preparing sample DNA and preparing Phix control!)

Library Pooling:

1. Label a fresh 1.5 ml microcentrifuge tube (Eppendorf, Germany) tube Pooled Amplicon Library (PAL)
2. Transfer the recalculated volume to have an equal concentration (±5 µL) of each library to be sequenced from the SGP plate into the PAL tube. Change tips between samples.
   NOTE:(!) in the end a minimal volume of 24 µL PAL is needed. Mix PAL tube by tapping or pipetting
3. Label a fresh 1.5 mL microcentrifuge tube (Eppendorf, Germany) tube DAL (Diluted Amplicon Library)
4. Add 576 µL of HT1 to the DAL tube
5. Transfer 24 µL of PAL to the DAL tube containing HT1. Mix by pipetting up and down.
6. Mix DAL by vortexing the tube at top speed
7. Using a heating block, incubate the DAL tube at 96°C for 2 min.
8. After incubation, invert DAL 1-2 times to mix and immediately place on ice.
9. Keep DAL on ice for 5 min.

Prepare PhiX control

1. Combine the following volumes to dilute the PhiX library to 4 nM:
   i. 2 µL 10nM PhiX library
   ii. 3 µL 10mM Tris-Cl, pH8.5 with 0.1% Tween 20
2. Combine the following volumes of 4nM PhiX library and freshly diluted 0.2N NaOH in a micro centrifuge tube:
   iii. 5 µL 4 nM PhiX library
   iv. 5 µL 0.2N NaOH
3. Vortex briefly to mix the 2nM PhiX library solution
4. Centrifuge the template solution to 280 x g for 1min
5. Incubate for 5min at room temperature to denature the PhiX library into single strands
6. Add the following volume of pre-chilled HT1 to the tube containing denatured PhiX library to result in a 20 pM PhiX library:
   v. 10 µL denatured PhiX library
   vi. 990 µL pre-chilled HT1
7. Dilute the denatured 20 pM PhiX library to 12.5 pM as follows
   vii. 375 µL 20 pM denatured PhiX library
   viii. 225 µL pre-chilled HT1
8. Invert several times to mix the solution

9. Discard the remaining dilution of 0.2N NaOH
10. Combine the following volumes of denatured PhiX control library and denatured sample library:
    ix. 60 µL denatured and diluted PhiX control
    x. 540 µL denatured and diluted sample library (DAL tube)
11. Set the combined sample library and PhiX control aside on ice until ready to load it onto the MiSeq reagent cartridge.

Loading Sample Libraries:

1. When the reagent cartridge is fully thawed and ready load prepared libraries onto the cartridge. Use a lab tissue to clean the foil seal covering the reservoir labelled "Load Samples"
2. Use a clean 1 mL pipette tip to pierce the foil seal covering the reservoir labelled "Load Samples"
3. Pipette 600 µL of combined sample library and PhiX control into the "Load Samples" reservoir. Avoid touching the foil seal as when dispensing the sample.
4. Proceed directly to the run setup steps using the MiSeq Control Software (MCS) interface.

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