Prevalence of aetiological pathogens of vaginal discharge and male urethritis syndromes in the Tshwane region

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Prevalence of aetiological pathogens of vaginal discharge and male urethritis syndromes in the Tshwane region

by

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Submitted in partial fulfilment of the requirements for the degree

MAGISTER SCIENTIAE

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I, the undersigned, declare that the dissertation hereby submitted to the University of Pretoria for the degree MSc (Medical Microbiology) and the work contained therein is my own original work and has not previously, in its entirety or in part, been submitted to any University for a degree.

Signed: _____ this _____ day of _____ 2015

"Man is a mine rich in germs of inestimable value.
Education can, alone, cause it to reveal its treasures and
enable mankind to benefit therefrom"
(Baha'i faith)

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LIST OF ABBREVIATIONS

4-SP	4-Sucrose phosphate
AMS	Antimicrobial susceptibility
AST	Antimicrobial susceptibility testing
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
CDC	Centres for Disease Control and Prevention
CDS	Coding sequence
CEACAM	Carcinoembryonic antigen-related cell adhesion molecules
CLSI	Clinical and Laboratory Standards Institute
CMV	Cytomegalovirus
DFA	Direct fluorescent antibodies staining
DGI	Disseminated gonococcal infection
DNA	Deoxyribonucleic acid
DUS	DNA uptake sequences
EB	Elementary body
EBV	Epstein-Barr virus
ECDC	European Centre for Disease Prevention and Control
ECM	Extracellular matrix
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
ESC	Extended-spectrum cephalosporin
FSW	Female sex worker
FVU	First-void urine
G+C	Guanine-plus-cytosine
HIV	Human immunodeficiency virus
HSPG	Heparin sulfate proteoglycans
HSV-1	Herpes simplex virus 1
HSV-2	Herpes simplex virus 2
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL	Interleukin

LAMP	Lipidassociated membrane proteins
LGV	Lymphogranuloma venereum
LOS	lipooligosaccharide
LPS	Lipopolysaccharide
MDR	Multidrug-resistant
MIC	Minimum inhibitory concentrations
MLSK	Macrolides, lincosamides, streptogramins, ketolides
MLST	Multilocus sequence typing
MOMP	Major outer membrane proteins
MUS	Male urethritis syndrome
NAAT	Nucleic acid amplification test
NAH	Nucleic acid hybridization test
NASBA	Nucleic acid sequence-based amplification
NCNGU	Nonchlamydial nongonococcal urethritis
NG-MAST	*Neisseria gonorrhoeae* multi-antigen sequence typing
NGU	Nongonococcal urethritis
nvCT	New variant of *C. trachomatis*
NYC	New York City agar
OM	Outer membrane
Opa	Opacity proteins
OPD	Out-patient department
ORF	Open reading frames
PBP	Penicillin binding proteins
PCR	Polymerase chain reactions
PFGE	Pulsed-field gel electrophoresis
PID	Pelvic inflammatory disease
PMN	Polymorphonuclear leukocyte
POC	Point-of-care
PPNG	Penicilinase-producing *N. gonorrhoeae*
QRNG	Quinolone-resistant *N. gonorrhoeae*
RB	Reticulate body
RE	Restriction endonuclease
RFLP	Restriction fragment length polymorphism

RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SCVS	Self-collected vaginal swab
SDA	Strand displacement amplification
ST	Sequence type
STD	Sexually transmitted disease
STI	Sexually transmitted infection
TLR	Toll-like receptor
TMA	Transcription-mediated amplification
TNF	Tumour necrosis factor
TTSS	Type III secretion system
UGA	Tryptophan codon
USA	United States of America
VDS	Vaginal discharge syndrome
VEC	Vaginal epithelial cell
WHO	World Health Organization
β-lactamase	Beta-lactamase

LIST OF PUBLICATIONS AND CONFERENCES

PUBLICATIONS

1. Osizigbo BI, MM Ehlers and MM Kock (2015) Molecular characterization and antimicrobial susceptibility profiles of *Neisseria gonorrhoeae* clinical isolates. In preparation for submission to: *Journal of Sexually Transmitted Infections*

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PREVALENCE OF AETIOLOGICAL PATHOGENS OF VAGINAL DISCHARGE AND MALE URETHRITIS SYNDROMES IN THE TSHWANE REGION

by

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SUMMARY

Sexually transmitted infections (STIs) continue to present major health, social and economic challenges in developing countries. The prevalence rate is higher in developing countries where STI treatment is less accessible. Sexually transmitted infections are caused by a large number of microbial agents that cause considerable morbidity, mortality and stigma worldwide.

In South Africa, the burden of STI is high and the most common STI presentations to the public healthcare are male urethritis syndrome (MUS) and vaginal discharge syndrome (VDS). Male urethritis account for over 60% of STIs annually, reported at healthcare facilities in the Gauteng province of South Africa. The main STI pathogens responsible for these two syndromes are *Chlamydia trachomatis, Neisseria gonorrhoeae* and *Trichomonas vaginalis*. Bacterial vaginosis, candidiasis, *Mycoplasma genitalium*, *Ureaplasma* species and viruses [Epstein-Barr virus (EBV), Herpes simplex virus (HSV-1, HSV-2), cytomegalovirus (CMV)] cause a small percentage of the vaginal discharge seen in clinical practice.

Sexually transmitted infections facilitate the transmission of the human immunodeficiency virus (HIV). This interaction may be a major factor in the rapid spread of HIV in sub-Saharan Africa. Hence, the control of STIs can lead to a substantial reduction in the transmission of HIV. However, antimicrobial resistance is making the clinical management of STIs such as gonorrhoea difficult.

The treatment options for gonococcal infections have reduced over time because of the emergence and spread of gonococci resistant to all antibiotics previously used as first-line empirical treatment. Currently, extended-spectrum cephalosporins (ESCs) are the only antibiotics recommended as first-line treatment of gonococcal infections. However, *N. gonorrhoeae* strains with reduced susceptibility or resistant to ESC and treatment failures with these agents has been reported. Thus, continued monitoring is mandatory to ensure that the optimal agent for empirical therapy is in use. Ideally, resistance monitoring should be combined with a highly discriminatory typing scheme; this will permit the detection of sexual networks and offers the potential for early detection of multidrug resistance (MDR) isolates.

In South Africa, STI treatment services are provided by both the public and private sectors. In the former, treatment for STIs is reliant on syndromic management. The success of this depends on early detection and constant monitoring of the aetiological pathogens. The aim of this study was to determine the prevalence of *N. gonorrhoeae, T. vaginalis, C. trachomatis* and *M. genitalium* as aetiological pathogens of MUS and VDS using a multiplex real-time polymerase chain reaction assay. Furthermore, the antimicrobial resistance of *N. gonorrhoeae* clinical isolates to eight antibiotics (penicillin, cefoxitin, ceftriaxone, cefpodoxime, tetracycline, ciprofloxacin, ofloxacin and spectinomycin) and the corresponding minimum inhibitory concentrations (MICs) were determined by using the Kirby Bauer disc diffusion and E-test methods. Finally, *N. gonorrhoeae* multi-antigen sequence typing (NG-MAST) was used to determine the genetic relatedness of *N. gonorrhoeae* clinical isolates.

The prevalence of MUS and VDS pathogens in this study was 57% (113/200). Pathogens responsible for urethritis were detected in 77% (48/62) of male participants while VDS were found in 47% (65/138) of the female participants. Resistance to quinolones increased by tenfold (7% in 2004 up to 70% in 2013) and it correlates with MDR isolates. All isolates were susceptible to third-generation cephalosporins. The NG-MAST method revealed novel as well as previously described sequence types (ST). The NG-MAST ST 11715, 11716, 11717 and 11718 are novel STs and have been registered at: (www.ngmast.net).

This study showed that *N. gonorrhoeae* is the most common cause of urethritis and vaginal discharge in the Tshwane region. The real-time multiplex PCR assay was rapid and suitable for the detection of all four pathogens at relatively low cost. This study supports cefixime as the current first-line treatment for *N. gonorrhoeae* in the Tshwane region, Gauteng, South

Africa. The findings of this study further support the testing of more than one pathogen in a PCR assay for the laboratory diagnosis of MUS and VDS.

Keywords: Antimicrobial susceptibility, Cephalosporins, *Chlamydia trachomatis,* Multiplex real-time PCR assay, *Mycoplama genitalium, Neisseria gonorrhoeae*, NG-MAST, self-collected urine, self-collected vaginal swab, *Trichomonas vaginalis*

CHAPTER 1

Introduction

Sexually transmitted infection (STI) is an illness that has a likelihood of transmission between humans by means of human sexual activity, including vaginal intercourse, oral sex and anal sex (CDC, 2010). Sexually transmitted infections are a major health problem globally (Mckechnie *et al.,* 2009). Worldwide, an estimated 340 million cases of curable STIs, which include chlamydial, gonorrhoea, trichomoniasis and syphilis, occur annually with an incidence that is increasing in many parts of the world (Mckechnie *et al.,* 2009).

Sexually transmitted infections (STIs) are usually characterised by an acute presentation that can progress to a chronic clinical condition (Samraa *et al.,* 2011). Both men and women can be affected by STIs (Samraa *et al.,* 2011). These infections constitute a hidden epidemic of enormous physical, psychological and economic consequences (Stellrecht *et al.,* 2004). According to the Centres for Disease Control and Prevention (CDC), an estimated 19 million new cases occur each year in the United States of America (USA), almost half of them in the 15 to 24 years age group (Forhan *et al.,* 2009).

Most STIs are asymptomatic but the consequences remain serious (Samraa *et al.,* 2011). Undiagnosed and untreated STIs can lead to long term complications (Peeling, 2009). While complications are rare in men, infections in women can lead to pelvic inflammatory disease and tubal infertility, a condition, which in some societies renders a woman worthless (Peeling *et al.,* 2006). Sexually transmitted infections can have serious adverse outcomes in pregnancy for both the woman and the foetus or infant (Peeling, 2009).

Prevention of sexually transmitted infections in this age of the human immunodeficiency virus (HIV) epidemic has taken on a renewed importance (Chua *et al.,* 2010). The rate of transmission of STIs varies greatly depending on social and economic situations (Joyee *et al.,* 2005). As such multiple strategies in addition to antibiotics and antivirals are needed to address this problem (Chua *et al.,* 2010). Furthermore, progress in vaccine development for most STIs is slow (Nusbaum *et al.,* 2004); at the moment, the only STI vaccine available is for high-risk types of human papillomavirus (Freeman *et al.,* 2006; Chua *et al.,* 2010). In the meantime pathogens, such as *Chlamydia trachomatis, Neisseria gonorrhoeae* and

Trichomonas vaginalis not only have the potential to cause significant morbidity on their own (Fleming *et al.,* 1999) but have been linked to the increased transmission of HIV (Joyee *et al.,* 2005).

Population screening and early treatment are critical for effective patient management to prevent the development of long term complications and to stop transmission (Peeling *et al.,* 2006). Scarce resources, long distances to healthcare facilities and lack of STI laboratory services are some of the limitations that infected individuals with symptoms have to endure in high burden areas before accessing STI treatment (Hawkes *et al.,* 1999).

The World Health Organization (WHO) recommends that countries with no or limited access to STI diagnostic services use syndromic management (WHO, 2001). This approach relies on the identification of consistent groups of symptoms and easily recognised signs and the provision of treatment that will cover the majority of and the most serious pathogens responsible for a given syndrome (WHO, 2001; De Jongh *et al.,* 2009). Syndromic management of STIs works well for urethral discharge, pelvic pain, urethritis and genital ulcer diseases (Lewis and Maruma, 2009).

In South Africa, the STI syndromic management approach was introduced to primary healthcare approximately 10 years ago (Lewis and Maruma, 2009). Flowcharts (algorithms) are produced, which guide healthcare workers in the correct implementation of the syndromic management of STIs (Lewis and Maruma, 2009). The established sexually transmitted pathogens implicated in the aetiology of urethritis and vaginal discharge includes: *Neisseria gonorrhoeae*, *C. trachomatis* (D to K strains) and *T. vaginalis*, whilst the pathogen status of both *Mycoplasma genitalium* and *Ureaplasma urealyticum* remains unclear (Pham-Kanter *et al.,* 1996; Ishihara *et al.,* 2004).

Evaluations of the WHO flowcharts have shown that the algorithm for vaginal discharge lacks both sensitivity and specificity for the identification of women with *C. trachomatis* and *N. gonorrhoeae* infections (Pepin *et al.,* 2004; Peeling, 2009). Syndromic management can result in as much as 98% overtreatment for *Chlamydia* and gonorrhoea in women presenting with vaginal discharge syndrome in countries where this treatment approach is used for the management of STIs (Peeling, 2009). The effect of overtreatment, the associated cost and the growing health burden of STIs have led to a need for rapid and reliable laboratory techniques

capable of simultaneously identifying multiple pathogens in a single clinical sample (Khan *et al.,* 2005; Lee *et al.,* 2007).

The current male urethritis syndrome (MUS) flowchart recommends a combination of cefixime and doxycycline as therapy for presumptive gonorrhoea and *C. trachomatis* infections respectively (Lewis *et al.,* 2009). According to the vaginal discharge syndrome (VDS) flowchart, an oral single 400 mg dose of cefixime (safe for both pregnant and lactating mothers) and amoxicillin (safe during pregnancy), given as oral 500 mg three times daily dose for one week are recommended for the treatment of gonorrhoea and *C. trachomatis* infections (Lewis and Maruma*,* 2009). This regimen is supplemented with 400 mg metronidazole as a single dose in non-pregnant women for the treatment of a potential infection with *T. vaginalis* (Lewis and Maruma*,* 2009; Zimba *et al.,* 2011). The disadvantage of amoxicillin is that it is ineffective against *M. genitalium* infection (Pépin *et al.,* 2005). The VDS guideline may need further revision should data in support of the role of *M. genitalium* as a significant reproductive tract pathogen in pregnant women emerge in the South African context (Haggerty *et al.,* 2006; Lewis and Maruma, 2009). Partner notification and treatment of male partners of infected women are important to avoid reinfection (Cudmore *et al.,* 2004).

This study is important especially in South Africa where syndromic management of STIs is strongly advocated; vital to this approach is the requirement for periodic surveillance to assess the microbiological causes of the various STI syndromes (aetiological surveillance); the prevalence of these agents, the antimicrobial susceptibility pattern and epidemiological profiling. The aim of this study was to determine the prevalence of the common aetiological pathogens for MUS, VDS and its co-infection in the Pretoria region. The result will also contribute to the understanding of the molecular epidemiology and the antimicrobial susceptibility pattern of *N. gonorrhoeae* in the Pretoria region.

Objectives

1 To collect urine specimens from 100 male participants and vaginal swabs from 100 female participants

2 To culture and compare the growth of *N. gonorrhoeae* from two specimens (urine and vaginal swabs) and culture *T. vaginalis* from vaginal swabs

3 To perform antimicrobial susceptibility testing and to determine the minimum inhibitory concentration of *N. gonorrhoeae* to eight antibiotics

4 To extract total genomic DNA from the vaginal swabs and urine specimens.

5 To extract DNA from *N. gonorrhoeae* pure culture for molecular typing.

6 To perform a multiplex real-time PCR using the total DNA extracted from urine specimens and vaginal swabs for the detection of *C. trachomatis*, *M. genitalium*, *N. gonorrhoeae* and *T. vaginalis.*

7 To compare the sensitivity, specificity and diagnostic accuracy of the multiplex PCR and the culture method for the detection of *N. gonorrhoeae* and *T. vaginalis*

8 To determine the clonal relatedness of the *N. gonorrhoeae* isolates

9 To perform data analysis

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Sexually transmitted infections (STIs) are defined as a group of communicable diseases which is transmitted predominantly by sexual contact (Alli *et al*., 2011). The number of organisms now known to be sexually transmitted includes about 20 pathogens (De Schryver and Meheus, 1990). Some of these agents [*Chlamydia trachomatis*, Herpes Simplex Virus (HSV), Human Papilloma Virus (HPV), Hepatitis B Virus (HBV), Human Immunodeficiency Virus (HIV), *Neisseria gonorrhoeae* and trichomoniasis] have replaced the classical "venereal diseases" both in importance and frequency as these agents are often more difficult to detect, treat and control (De Schryver and Meheus, 1990; Alli *et al*., 2011).

Neisseria gonorrhoeae is an exclusive human pathogen (WHO, 2011); that causes 106.1 million new cases of gonorrhoea infections annually globally with 21.1 million of these new cases occurring in Africa (WHO, 2012). It is the aetiological agent of gonorrhoea, which can cause cervicitis, urethritis, proctitis and pelvic inflammatory disease (PID) (Fleming and Wasserheit, 1999). Untreated *N. gonorrhoeae* infections can result in long-term complications, such as infertility, ectopic pregnancy and chronic pelvic pain and adverse outcomes of pregnancy (Rottingen *et al*., 2001).

Identification of cases and effective treatment with antibiotics are the main approaches for the prevention and control of gonorrhoea (Tapsall, 2001). However, there is a disturbing trend worldwide regarding the rising incidence of gonorrhoea in many countries (Unemo and Dillon, 2011). In addition to the increasing prevalence, strains that are resistant to inexpensive and widely available antibiotics are also on the increase (Tapsall *et al*., 2009; Bolan *et al*., 2012).

Consequently gonorrhoea may become untreatable in certain situations and is fast becoming a “superbug” (Unemo and Dillon, 2011). In 2007, the Centres for Disease Control and Prevention (CDC) added *N. gonorrhoeae* to the list of “superbugs” (Zalucki, *et al*., 2012). International efforts to gather information on the emerging trends in antimicrobial susceptibility together with regional, national and international surveillance of the

epidemiological characteristics and spread of *N. gonorrhoeae* have become a public health priority (Unemo and Dillon, 2011).

Chlamydia trachomatis is an obligate intracellular human pathogen and one of the most common STIs worldwide (Venter *et al*., 2011). Global prevalence has been estimated at 105.7 million annually, with about 9.2 million occurring in Africa (WHO, 2012). Sexually acquired chlamydial infection may manifest as cervicitis, epididymo-orchitis, PID and urethritis (serovars D to K) or as genital ulceration (serovars L1 to L3) (Laga *et al*., 1993). The spread of chlamydial serovars D to K has been referred to as the 'silent epidemic' because approximately half of the infections in males and up to 70% in females are asymptomatic (Laga *et al*., 1993; Anttila *et al*., 2001; Lorimer and McDaid, 2013). Acute *C. trachomatis* infection is easy to treat, yet the majority of chlamydial infections remain untreated because of the asymptomatic carriage of the bacteria (Wiesenfeld *et al*., 2002).

Trichomonas vaginalis is the most common cause of non-viral STIs in the world, with 174 million estimated new cases annually (Tambekou *et al*., 2009). In sub-Saharan Africa, the incidence is estimated at 32 million (Tambekou *et al*., 2009). The infection is asymptomatic in about 50% of infected women and in over 90% of men, thus re-infection and re-exposure is problematic (Tambekou *et al*., 2009). Trichomoniasis is often considered as a relatively benign infection, but given its association with other STIs, human immunodeficiency virus (HIV) and preterm delivery, it is a significant public health problem (Miller *et al*., 2005; Sutton *et al*., 2007). Metronidazole is the drug of choice for the treatment of *T. vaginalis* infections, but treatment failures and drug resistance have been reported (Gehrig and Efferth, 2009).

Mycoplasma genitalium is a flask-shaped *Eubacterium* that lacks a true cell wall and has the smallest genome among self-replicating free-living organisms (Gambini *et al*., 2000). *Mycoplasma genitalium* is sexually transmitted (Ross and Jensen, 2006) and has a role in approximately 25% of cases of nongonococcal urethritis and prostatitis in men and is implicated in cervicitis (Anagrius *et al*., 2005), endometritis, PID and infertility (Yoshida *et al*., 2002; Simms *et al*., 2003; Cohen *et al*., 2005). *Mycoplasma genitalium* grows very slowly hence, a prolonged course of therapy (five to ten days) is appropriate in symptomatic patients in whom the bacterium has been detected (Ross and Jensen, 2006). A number of different antibiotics have been used to treat *M. genitalium* infections with varying degrees of

success (Ross and Jensen, 2006). Macrolides, in particular azithromycin, offer the best chance of cure with a 84% clearance in men with *M. genitalium* urethritis (Mena *et al*., 2009).

Sexually transmitted infections (STIs) increase the risk of acquiring HIV (Wasserheit, 1992). The control of STIs is recommended for HIV prevention (Mavedzenge *et al*., 2010). In the late 1990s the South African health department introduced the STI syndromic management approach into the primary health care setting (Lewis and Maruma, 2009). This approach aims to treat the common causes of STI syndromes through the use of specific treatment algorithms (Mhlongo *et al*., 2010). Syndromic management is cost effective, because it allows caregivers to treat the majority of STI patients without the need for laboratory-based diagnostics (Mhlongo *et al*., 2010). Surveillance is a vital component of syndromic management and should generate data on clinically diagnosed STI syndromes, syndrome aetiology, antimicrobial resistance and pathogen epidemiology (De Jongh *et al*., 2009).

2.2 Aetiological pathogens of vaginal discharge and male urethritis syndromes

Urethritis is characterised by urethral inflammation; this can result from infection or immune sources (Tsai and Li, 2013). Urethritis of infectious origin is usually acquired through a STI and shows similar symptoms and signs regardless of causative pathogen (Tsai and Li, 2013). The most common presentations are mucopurulent or purulent discharge from the urethral meatus, dysuria, itching or a burning sensation in the urethra (Le Roux *et al*., 2010; Tsai and Li, 2013). Urethritis is classified according to the STI causing the infection (Alli *et al*., 2011). In men, gonoccocal urethritis (caused by *N. gonorrhoeae*) presents as an acute urethritis (Edwards and Butler, 2011).

Nongonococcal urethritis (NGU) is a urethral inflammation that is not caused by *N. gonorrhoeae* infection and is a common type of urethritis in men (Schwebke *et al* 2011). It accounts for 10% to 30% of new male cases in STI clinics (Handsfield, 2011; Tsai and Li, 2013). *Chlamydia trachomatis*, genital mycoplasmas, viruses and protozoan parasites are pathogens associated with NGU (Martin, 2008), but *C. trachomatis* is the most prevalent and well-established (Bradshaw *et al*., 2006; Tsai and Li, 2013).

Urethritis caused by agents other than *N. gonorrhoeae* and *C. trachomatis* is called nonchlamydial NGU (NCNGU) (Bernsson *et al*., 2010). *Mycoplasma genitalium* and *Ureaplasma urealyticum* (biovar 2, subtype 1) are well-known causes of NCNGU (Bernsson *et al*., 2010). Viruses, such as EBV, HSV-1, HSV-2, CMV and adenovirus have been

implicated as aetiological pathogens of NCNGU (Bernsson *et al*., 2010). However, pathogens still cannot be detected in most cases of NCNGU (Bradshaw *et al*., 2006; Workowski and Berman, 2010).

There are two types of vaginal discharge; physiological and pathological vaginal discharge (Mitchell, 2004). Symptomatic vaginal discharge results from inflammation due to infection of the vaginal mucosa (Rekha and Jyothi, 2010). Infection caused by bacterial vaginosis, *Candida* species, *C. trachomatis*, HSV, *N. gonorrhoeae* and *T. vaginalis* can lead to vaginal discharge (Yusuf *et al*., 2011). Pathological discharge caused by anyone of these agents is referred to as abnormal vaginal discharge (Yusuf *et al*., 2011).

Vaginal discharge is a poor predictor of the presence of a STI (Mitchell, 2004). Nevertheless, abnormal vaginal discharge often prompts women to seek screening for STIs (Mitchell, 2004). This is because, abnormal vaginal discharge predisposes to morbidity in the form of endometriosis, infertility, cuff cellulitis, pregnancy loss, preterm labour and PID (Rekha and Jyothi, 2010).

2.3 *Neisseria gonorrhoeae* a "superbug"

Gonorrhoea, caused by *N. gonorrhoeae*, is the second most prevalent bacterial sexually transmitted infection globally (Ohnishi *et al*., 2011). The disease is associated with high morbidity and socioeconomic consequences and remains a public health problem worldwide (Ohnishi *et al*., 2011). In the absence of a vaccine, appropriate diagnostics and antimicrobial therapy are the key elements for reduction and control of gonorrhoea (Tapsall *et al*., 2009). Over time, the hope that there would be a never-ending supply of effective antibiotics that could be used in single dose for the treatment of gonococcal infections has steadily diminished (Dillon, 2011). This is due to the progressive development of resistance in isolates of *N. gonorrhoeae* to antimicrobial agents used to treat gonococcal infections (Lewis, 2010). However, the threat of potentially untreatable gonococcal infections is not new (Dillon, 2011). In the early 1990s, this possibility was recognized by the World Health Organization (WHO), the acknowledgment led to the formation of the first Gonococcal Antimicrobial Surveillance Program (GASP) initiative (WHO 1990; Dillon, 2011). Subsequently in the mid-2007 the CDC listed *N. gonorrhoeae* as a "superbug" (Zalucki, *et al*., 2012). *Neisseria gonorrhoeae* may soon become a true superbug that initiates a future era of untreatable gonorrhoea (Ohnishi *et al*., 2011).

2.3.1 History of *N. gonorrhoeae*

The term gonorrhoea can be credited to the second century Greek physician Galen who named the disease symptoms a "flow of seed" (Unemo and Shafer, 2011). *Neisseria gonorrhoeae* is an ancient disease, in this regard a passage in the Old Testament (Leviticus 15:1–3), warns: "When any man has a bodily discharge, the discharge is unclean," this gives reason to believe that gonorrhoea is an ancient disease and has successfully adapted itself to existing with humans (Unemo and Shafer, 2011).

The causative agent of gonorrhoea, *N. gonorrhoeae*, was described in 1879 by Albert Neisser (Tønjum, 2005a). In 1885, the organism was grown in pure culture (Hook and Handsfeild, 1999). Its aetiological relationship to human disease was determined using human volunteers in order to meet the experimental requirements of Koch's postulates (Hook and Handsfeild, 1999; Hobbs *et al*., 2011). Till date, gonococcal infection remains a major global health problem, as over 90 million cases are reported annually worldwide (Zalucki *et al*., 2012).

2.3.2 Classification of *N. gonorrhoeae*

Neisseria gonorrhoeae bacteria are prokaryotes and are grouped in the domain *Bacteria* (Garrity *et al*., 2004). The domain contains 24 phyla of which *N. gonorrhoeae* are located in the *Proteobacteria* phylum (Euzéby, 2012). The order *Neisseriales* is made up of 32 genera exhibiting a wide range of growth requirements, habitat and morphologies (Tønjum 2005b; Euzéby, 2012). This order contains a group of Gram negative, non-spore forming, aerobic and mesophilic bacteria (Adeolu and Gupta, 2013). The Genus *Neisseria* includes at least 25 species (Table 2.1) based on 16S rRNA gene sequence information (Hung and Christodoulides, 2013).

Table 2.1: **Scientific classification of *N. gonorrhoeae* (Garrity *et al*., 2004; Euzéby, 2012)**

Taxonomic rank	Scientific name
Domain	*Bacteria*
Phylum	*Proteobacteria*
Class	*Beta-proteobacteria*
Order	*Neisseriales*
Family	*Neisseriaceae*
Genus	*Neisseria*
Species	*N. gonorrhoeae, N. canis, N. caviae, N. cinerea* *N. lactamica, N. dentiae, N. meningitidis, etc*
Binomial name	***Neisseria gonorrhoeae***

The genus *Neisseria* includes Gram-negative, oxidase positive diplococci, many of which are harmless commensal inhabitants of the dental and mucosal surfaces of humans (Zaura et al., 2009). Species of the genus *Neisseria* are usually distinguished based on their phenotypic properties and carbohydrate utilisation patterns (Bennett *et al*., 2012). This genus contains two important human pathogens that cause very different diseases (Adeolu and Gupta, 2013). *Neisseria gonorrhoeae*, the gonococcus, which causes gonorrhoea and, occasionally, disseminated infections and *Neisseria meningitidis*, the meningococcus, which causes meningitis and septicaemia (Bennett *et al*., 2012; Adeolu and Gupta, 2013).

2.3.3 General characteristics of *N. gonorrhoeae*

Neisseria gonorrhoeae is an aerobic, Gram-negative, non-motile, non-spore-forming coccus (Adeolu and Gupta, 2013). *Neisseria gonorrhoeae* has a cell size of 0.6 μm to 1.9 μm in diameter, occurring singly but often in pairs with adjacent sides flattened (Tønjum, 2005a). These organisms are structurally differentiated from other cocci by their 'kidney-shaped' diplococcal forms (Tønjum, 2005a). *Neisseria gonorrhoeae* can utilise glucose, lactate or pyruvate as sole carbon sources, but cannot metabolise other carbohydrates (Apicella, 2005). Its protein and carbohydrate surface antigens lack capsular polysaccharides (Feavers and Maiden, 1998). *Neisseria*'s cell membrane is made up of outer membrane proteins, lipids,

lipopolysaccharides and has an inner peptidoglycan matrix (Edward and Butler, 2011). Porin proteins cover the cell membrane and forms aqueous channels that allow the exchange of antibiotics, dyes, ions and other larger compounds to occur (Brooks and Donegan, 1985; Cooper *et al*., 2012). *Neisseria gonorrhoeae* forms opaque colonies *in vitro* and has the ability to form biofilms on glass surfaces, primary and immortalised urethral and cervical epithelial cells (Youssef *et al.,* 2009; Philips *et al*., 2012).

2.3.3.1 Microbiological characteristics of *N. gonorrhoeae*

Neisseria gonorrhoeae is a fastidious organism that exhibits complex growth requirements when cultured *in vitro* (Hansfield, 2005). It grows on a chocolate agar base media, which contains antibiotics that inhibit the growth of yeast and other bacteria (Spence *et al.*, 2008). Gonococcal medium is typically supplemented with Kellogg's supplements and 0.042% $NaHCO_3$ or IsoVitalex (Becton-Dickinson, Franklin Lakes, NJ) to enhance the growth of gonococci on plates (Spence *et al.*, 2008; Kohler *et al*., 2013). The following rich media are used for the isolation of *N. gonorrhoeae*: Thayer-Martin, modified Thayer-Martin, Lewis-Martin, Difco GC medium and New York City agar (NYC) (WHO, 2013).

Neisseria gonorrhoeae is capnophilic, hence it prefer enhanced concentrations of 3% to 7% carbon dioxide (CO_2) for growth, this can be provided by growth on a solid medium in a CO_2-incubator or candle jar (Spence *et al.*, 2008; WHO, 2013). *Neisseria gonorrhoeae* is cultured at 37^{o}C and does not survive at temperatures below 25°C (Spence *et al.*, 2008). Bacterial colonies appear on plates within 24 to 48 h of inoculation (WHO, 2013), but viability is rapidly lost due to autolysis after depletion of glucose from the medium (Spence *et al.*, 2008). Colony size and appearance may vary from 0.5 to 1 mm in diameter and from grey to white, transparent to opaque and convex to flat (depending on gonococcal strain and culture medium) (WHO, 2013). After further incubation, it may reach 3 mm in diameter and become less smooth; frequently a mixture of different colony types appears on a plate (WHO, 2013).

2.3.3.2 Genomic characteristics of *N. gonorrhoeae*

Neisseria gonorrhoeae is a highly competent bacterium that has the capacity to alter its phenotypic and genotypic characteristics to adapt to host influences (Whiley *et al*., 2006). It is naturally competent and is able to take up exogenous deoxyribonucleic acid (DNA) (Hamilton and Dillard, 2006). The gained DNA maybe incorporated and used to transpose novel functions into its genome (Hamilton and Dillard, 2006; Helena and Thomson, 2013).

This phenomenon is referred to as natural transformation (Hamilton and Dillard, 2006). Natural transformation is the only mechanism by which horizontal transfer of chromosomal markers occurs in *N. gonorrhoeae*, hence it accounts for the emergence of mosaic alleles for single copy genes of the gonococcal genome (Hamilton *et al*., 2005; Hamilton and Dillard, 2006).

In general the *N. gonorrhoeae* genome has 2 662 coding sequences (CDSs) and a variable plasmid complement (Helena and Thomson, 2013). The plasmids associated with the strains of *N. gonorrhoeae* are: The small cryptic plasmid of 4.2 kb and larger conjugative plasmids, which promote its own transfer between strains and may carry determinants of antibiotic resistance (Snyder *et al*., 2005). The genome contains 12 *opa* loci that code for opacity protein genes (Youssef *et al.,* 2009). Over 1 900 copies of specialized repeat elements called DNA uptake sequences (DUSs), which is about 10 bp to 12 bp in size are present in high density throughout the *N. gonorrhoeae* genome (Helena and Thomson, 2013). The *N. gonorrhoeae* DUSs are found in closely related commensal and pathogenic *Neisseria* species including *Neisseria meningitidis*, which provides an ideal opportunity for genetic exchange (Marri *et al*., 2010).

2.3.4 Virulence factors and pathogenesis of *N. gonorrhoeae*

Neisseria gonorrhoeae is the only species among the genus *Neisseria* that is always pathogenic (Cornelissen, 2011). Strains of this species infect surfaces of the mucosal cells in the cervix, urethra, rectum, oro-nasopharynx, causing symptomatic or asymptomatic infections (Cornelissen, 2011). In order to colonise host cells successfully, *N. gonorrhoeae* need to establish and maintain an association with host cell surfaces (Hung and Christodoulides, 2013). Hence, colonisation involves adhesion, bacterial aggregation, microcolony and biofilm formation and the avoidance of host immunity (Hung and Christodoulides, 2013).

The surface structures mediating adherence of *N. gonorrhoeae* to host epithelial cells are called adhesins (Hung and Christodoulides, 2013). The *N. gonorrhoeae* adhesins include: the major outer membrane protein (porins); the opacity-associated proteins (Opa and Opc), the most abundant protein adhesins in the outer membrane (OM); lipooligosaccharide (LOS) and the pili (Edward and Butler, 2011; Hung and Christodoulides, 2013). The opacity protein adheres to heparin sulfate proteoglycans (HSPGs) (Edward and Butler, 2011). The Opa

variants are able to bind to different receptors of the carcinoembryonic antigen-related cell adhesion molecules (CEACAM) family on epithelial cells, neutrophils, T and B lymphocytes, influencing the innate and adaptive immune responses (Edward and Butler, 2011).

The LOS plays important roles in serum resistance as well as resistance to cationic antimicrobial peptides (Cornelissen, 2011). The Pili are involved in the attachment of the gonococcus to host epithelial cells (Hung and Christodoulides, 2013). *Neisseria gonorrhoeae* expresses type IV-A pili, which plays a role in mediating important cellular functions, such as biofilm formation, cell signalling, DNA uptake by natural transformation, host-cell adhesion and surface motility (Hung and Christodoulides, 2013).

Neisseria gonorrhoeae evades killing by human complement by using its porin molecules to bind complement down-regulatory protein in other to maintain infection in host cells (Ngampasutadola *et al*., 2008). Other mechanisms used by *N. gonorrhoeae* to evade killing by host complement include, antigenic phase variation of the OMP and LOS sialylation (Johnson and Criss, 2011; Srikhanta *et al*., 2010; Anderson and Seifert, 2013).

In general, gonococcus infection often leads to inflammation and polymorphonuclear leukocyte (PMN) influx (Virji, 2009). *Neisseria gonorrhoeae* engulfed by PMN alongside with infected epithelial cells are secreted in PMN-rich exudates from the mucosal surface to the urethral lumen and it is often seen as discharge (Edwards and Apicella*.,* 2004; Virji, 2009). Tumour necrosis factor (TNF) from phagocytes and gonococcal products, such as peptidoglycan and lipopolysaccharide (LPS), also causes toxic damage to ciliated epithelial cells of mucosal surfaces (Virji, 2009).

2.3.5 Immunological response to *N. gonorrhoeae* infections

The clinical symptoms of gonococcal infections are caused primarily by the intense inflammatory infiltrate of neutrophils responding to cytokines expressed by induction of the innate immune system (Cooper *et al*., 2012). Gonococcal infection can be acquired repeatedly with no apparent reduction in severity or length of disease (Russell and Hook, 2009). Hence, recovery from each incident of infection does not leave an effective protective immunity against the organism (Liu and Russell, 2011).

Neisseria gonorrhoeae infection triggers a potent local inflammatory response driven by PMNs (Johnson and Criss, 2011). Polymorphonuclear leucocytes are phagocytic cells that are

important components of the host innate immune response and are the first responders to bacterial and fungal infections (Johnson and Criss, 2011). Polymorphonuclear leucocytes possess a range of components to combat microorganisms, including the production of reactive oxygen species and release of cell degrading enzymes and antimicrobial peptides (Johnson and Criss, 2011). Activation of the complement system by *N. gonorrhoeae* will lead to proteolytic cascades that will terminate in opsonisation and lysis of the pathogen as well as in the generation of the classical inflammatory response through the production of potent pro-inflammatory molecules (Dunkelberger and Song, 2010).

2.3.6 Clinical manifestations of *N. gonorrhoeae* infections

The most common presentation in men is a purulent urethral discharge and dysuria (Bignell, 2009). Cervical infection is common in women and symptoms result in endocervical and urethral findings (Lewis *et al.,* 1999). These include increased or altered vaginal discharge, inter-menstrual bleeding, dysuria and menorrhagia (Lewis *et al.,* 1999; Bignell, 2009). Physical findings in women include cervicitis with mucus-purulent drainage from the cervix (Bignell, 2009). The cervix tends to bleed easily when rubbed with a cotton-tipped swab (Miller, 2006). Periurethral oedema and purulent discharge from the cervix without vaginitis is not uncommon (Bernstein *et al.,* 2009). It can present as vulvovaginitis and Bartholin's abscess in prepubescent females (Bernstein *et al.,* 2009). Asymptomatic genital tract infection occurs in up to 10% and 50% of men and women respectively (Bignell, 2009).

Deeper structures, such as the pelvic and the fallopian tubes can be infected with untreated gonococcal disease (Workowski and Levine, 2002). Ten to twenty percent of women with gonorrhoea develop ascending infection that leads to acute salpingitis with or without endometritis (Miller, 2006). This condition is also known as pelvic inflammatory disease (PID) (Workowski and Levine, 2002). The clinical symptoms may range from no symptoms to severe abdominal pain with a high fever (Miller, 2006). Pelvic inflammatory disease can negatively affect fertility (Workowski and Levine, 2002). Up to fifty percent of patients who had suffered three or more episodes of PID will not be able to fall pregnant (Miller, 2006).

Untreated gonococcal infection can spread to deeper structures and disseminate, resulting in disseminated gonococcal infection (DGI) (Peters *et al.,* 2011). Disseminated gonococcal infection can cause bacteraemia, which occasionally lead to chronic joint infections and sepsis (Walker and Sweet, 2011). Disseminated gonorrhoea frequently results in petechial or

pustular acral skin lesions, asymmetrical arthralgia, tenosynovitis or septic arthritis (Walker and Sweet, 2011). Oropharyngeal infection with *N. gonorrhoeae* always coexists with a genital infection and will follow oral-genital sexual exposure (Peters *et al.,* 2011). In females and homosexual males, this type of exposure will present as pharyngitis with erythema (Bernstein *et al.,* 2009).

Ophthalmia neonatorum is an acute mucopurulent conjunctivitis caused by *N. gonorrhoeae*, it is principally an infection acquired during vaginal delivery (Zuppa *et al*., 2011). Neonatal conjunctivitis often follows intrapartum contamination of the eye with cervical secretion if the mother had an unrecognised and untreated genital infection acquired by sexual contact (Palafox *et al.,* 2011). Untreated disease can lead to chronic infection lasting many months; vision loss is usually due to eyelid scarring (Palafox *et al.,* 2011; Zuppa *et al* 2011).

2.3.7 *Neisseria gonorrhoeae* and HIV

Epidemiological, clinical and observational studies have shown that STIs significantly increase the chance of HIV transmission (Galvin and Cohen, 2004; Jin *et al*., 2010). The underlying mechanisms involved are not clear (Simon *et al*., 2006).

Neisseria gonorrhoeae is one of the prevalent bacterial STIs in people living with HIV-1 (Page *et al*., 2008; Mlisana, 2012). Symptomatic *N. gonorrhoeae* infection is associated with increased viral shedding and detection of HIV-1 from genital secretions of men and women (Malotta *et al*., 2013). Viral shedding is associated with a two to five fold increase in male-to-female HIV-1 transmission rates (Malotta *et al*., 2013). The increase in the transmission rate is thought to emanate from the ability of *N. gonorrhoeae* to inhibit the maturation of dendritic cells and its ability to prime HIV-specific immune responses (Sheunga *et al*., 2008; Malotta *et al*., 2013).

2.3.8 Diagnosis of *N. gonorrhoeae*

Microscopy using Gram staining offers good sensitivity (95%) and specificity as a rapid diagnostic test for *N. gonorrhoeae* in symptomatic men with urethral discharge (Bignell, 2009). Microscopy has poor sensitivity (55%) in asymptomatic men and in identifying endocervical (55%) or rectal infection (40%) and cannot be recommended as a test of exclusion in these situations (Bignell, 2009). Microscopic examination of a Gram stain is one method that offers the advantage of point-of-care (POC) diagnosis, facilitating early treatment

(Koumans *et al*., 1998; WHO, 2013). The optical immunoassay (OIA) is another POC test for *N. gonorrhoeae* (Gaydos, 2006). This test is based on a solid phase enzyme immunoassay, which uses a cartridge, reagent mix and a wick (Gaydos, 2006). The sensitivity and specificity of the OIA test for symptomatic female endocervical swabs is 70.7% and 99.4% respectively. For symptomatic male urines is 93.2% and 97.5% respectively (Gaydos, 2006).

Before the 1980s, the options for testing for gonorrhoea were limited; culture with the use of selective medium was wildly used (Bignell *et al.,* 2006). This method was cheap, highly sensitive and specific in laboratories with good quality control measures (Koumans *et al*., 1998). There are two approaches available for culturing *N. gonorrhoeae* (Harryman *et al.,* 2012); direct plating at time of specimen collection or use of transport media to transfer specimens to the laboratory where direct plating is subsequently performed (Harryman *et al.,* 2012). Under optimal conditions both approaches may have a similar sensitivity (Olsen *et al*., 1999). However, with the transport media, failure to store at 2°C to 8°C once taken and a delay longer than 6 h before plating out on growth media can adversely affect the latter approach (Olsen *et al*., 1999).

Selective culture media containing antimicrobials are recommended for culturing *N. gonorrhoeae* because of its fastidious nature (Kent *et al*., 2005; Bignell, 2009). Culture is appropriate for endocervical, urethral, rectal and pharyngeal specimens (Jephcott, 1997; Bignell *et al*., 2006). Identification of *N. gonorrhoeae* by culture remains the "gold standard" for test evaluation and routine screening because of its high sensitivity and specificity (Hopkins *et al*., 2010). In laboratories with good quality control measures, the sensitivity of culture may range from 85% to 95% (Koumans *et al*., 1998). *Neisseria gonorrhoeae* identification by culture offers the advantage of retention of the organism for other tests (Harryman *et al.,* 2012). These advantages include the determination of antimicrobial susceptibility testing and genotyping (Harryman *et al.,* 2012). Some of the disadvantages of this method are; the fastidiousness of the organism, specimen transport, strict quality control at each step and the length of time required from specimen collection to reporting of results (Koumans *et al*., 1998; WHO, 2013).

Nucleic acid amplification tests (NAATs) have high sensitivity and specificity compared with culture for the detection of *N. gonorrhoeae* (Van Dyck *et al*., 2001; Shipitsyna *et al*., 2008). The main types of NAATs that has used for the detection of *N. gonorrhoeae* include

transcription-mediated amplification (TMA), polymerase chain reactions (PCR), real-time nucleic acid sequence-based amplification (NASBA) and strand displacement amplification (SDA) (Lindan *et al*., 2005; Shipitsyna *et al*., 2008; Le Roux *et al*., 2010; Walker and Sweet, 2011). All these methods detect and copy gonococcal DNA to enhance detection (Walker and Sweet, 2011). The amplification principles, mode of detection and the nucleic acid target vary between different NAAT methods (WHO, 2013). Nucleic acid amplification tests are approved for use with urine, urethral, endocervical samples and vaginal swabs by the US Food and Drug Administration (FDA) (Walker and Sweet, 2011). The disadvantages of NAATs include; high cost and the risk of cross contamination with highly sensitive amplification methods (Lai-King and Martin, 2005; Lindan *et al*., 2005).

2.3.9 Treatment of *N. gonorrhoeae* infections

The treatment of gonorrhoea is complicated by the ability of *N. gonorrhoeae* to develop resistance to antibiotic treatment (Newman *et al*., 2007). Resistance to all antibiotic treatments currently recommended for the treatment of gonorrhoea has been documented (Lo *et al*., 2012). The 2010 STI treatment guideline was designed to treat gonorrhoea by anatomic site of infection (CDC, 2010). Ceftriaxone has been shown to cure 99.2% of uncomplicated urogenital and anorectal infection (Pandori *et al*., 2009) and 98.9% of pharyngeal infections (Yokoi *et al*., 2007; Pandori *et al*., 2009).

A single dose of 125 mg ceftriaxone is recommended for the treatment of lower genital and anorectal infections (Newman *et al*., 2007), a double dose is recommended to reduce development of resistance and to cover for unrecognised oropharyngeal infection (Unemo *et al*., 2011; Walker and Sweet, 2011). Other injectable cephalosporins recommended for gonococcal treatment include ceftizoxime, cefoxitin and cefotaxime (Walker and Sweet, 2011). The only recommended oral choice for gonorrhoea is cefixime, it has a cure rate of 97.5% for uncomplicated urogenital and anorectal infections and 92.3% for pharyngeal gonococcal infections (Walker and Sweet, 2011). Saline lavages of the infected eye and treatment with a high-dose of ceftriaxone are recommended for the treatment of adult gonococcal conjunctivitis (McLean *et al*., 2004).

2.3.10 Antibiotic resistance mechanisms to *N. gonorrhoeae*

Antimicrobial resistance is an increasing problem in *N. gonorrhoeae* treatment worldwide (De Vries *et al.,* 2009). The CDC and the WHO recommended discontinuing the use of an antibiotic for patient management, as soon as the prevalence of antibiotic resistant strains of circulating pathogens in a patient population exceeds 5% (Tapsall, 2001; Workowski *et al.,* 2008). Heritable changes, including resistance to antibiotics in gonococci develops due to spontaneous gene mutation and acquisition, which are selected due to antibiotic pressure (Unemo and Shafer, 2011). Resistance determinants can be donated to other gonococci once established mainly by transformation, which substantially facilitates spread of resistance alleles (Unemo and Shafer, 2011). Exchange of inherited plasmids called conjugal transfer of DNA (gene) can occur between different *N. gonorrhoeae* strains, as well as with *N. meningitidis*, *Haemophilus influenzae* and *Escherichia coli* (Bäckman *et al*., 2000; Unemo and Shafer, 2011).

Penicillin and other β-lactam antibiotics exert their action by competitive inhibition of the penicillin binding proteins (PBPs) (Tomberg *et al*., 2013). Modifications in PBP-2 and PBP-1 decrease their affinity for the penicillins and thus the susceptibility of the organism (Unemo and Shafer, 2011). Changes in other loci such as multiple transferrable resistance regulator (*mtr*R) and *pen*B produce additive effects (Tomberg *et al*., 2013). The *mtr*R locus increases the expression of the MtrC-MtrD-MtrE efflux pump (Veal *et al*., 2002; Tomberg *et al*., 2013). This determinant mediates resistance to a wide range of antibiotics, through an active efflux pump system (Tomberg *et al*., 2013).

The mechanism involved with decreased susceptibility and resistance *in vitro* to third-generation extended-spectrum cephalosporins (ESCs) by *N. gonorrhoeae* is the alteration of the *pen*A gene encoding PBP 2 (Lewis *et al*., 2013). The central mechanism of fluoroquinolone (ciprofloxacin) resistance involves mutation of the *gyr*A gene (particularly in codon 91 and 95) that encodes an altered subunit GyrA of the enzyme DNA gyrase (Deguchi *et al.,* 1996; Sethi *et al*., 2013). This variation makes the binding of fluoroquinolones and hence the inhibition of cell replication impossible (Deguchi *et al.,* 2010). Resistance to spectinomycin results from production of adenyltransferases that inactivate the drug (Unemo *et al*., 2013). Alterations of specific amino acids in loop 2 of the 30S ribosomal protein S5 (encoded by *rpsE*) and mutations in the spectinomycin binding region will also produce

resistance to spectinomycin (Criswell *et al*., 2006 Kirthi *et al*., 2006; Kehrenberg and Schwarz, 2007).

2.3.11 Typing methods for *Neisseria gonorrhoeae*

Neisseria gonorrhoeae is highly variable in its genotype and phenotype (Fredlund *et al*., 2004). This genetic difference has been used to characterise gonococcal strains, thereby identifying patterns of specific strain transmission and guiding control efforts (Unemo *et al*., 2004; Liao *et al*., 2008). Many typing methods have been developed for *N. gonorrhoeae* based on phenotypic and genotypic characteristics of the bacterium (Liao *et al*., 2008). Traditional, non-DNA-based phenotypic typing methods include; auxotyping and serotyping (ECDC, 2012). Phenotypic characterisation methods are laborious, lack reproducibility, it also lack sensitivity for describing isolates appropriately and it is subjective in the interpretation of results (Unemo and Dillon, 2011). Deoxyribonucleic acid-based typing methods (genotypic typing) have better discriminatory power than the traditional phenotypic methods and result is reproducible (Unemo and Dillon, 2011).

The following genotypic characterisation method; Opa typing; pulsed-field gel electrophoresis (PFGE); restriction fragment length polymorphism (RFLP) and ribotyping are based on restriction endonuclease (RE) analysis (Kolader *et al*., 2006; Khaki *et al*., 2009; Unemo and Dillon, 2011; Sathirareuangchai *et al*., 2014). These methods consists of extraction of DNA from the organism and digesting the DNA *in vitro* with an appropriate restriction enzyme, followed by electrophoretic separation of the DNA fragments in either agarose gel or polyacrylamide gel (Chen *et al*., 2008; Unemo and Dillon, 2011).

Deoxyribonucleic acid sequence-based typing techniques used for the characterisation of *N. gonorrhoeae* include: *por*B-based DNA sequence typing, *N. gonorrhoeae* multi-antigen sequence typing (NG-MAST) and Multilocus sequence typing (MLST) (Bennett *et al*., 2007; Sethi *et al*., 2013; Whiley *et al*., 2013). These DNA sequence-based typing methods are faster, less laborious and all *N. gonorrhoeae* strains are typeable. Results are reproducible and interlaboratory comparison is possible based on DNA sequence (Unemo and Dillon, 2011; Sethi *et al*., 2013). A database that assigns a sequence type (ST) number based on the unique DNA sequence has been established for each technique, thereby allowing international standardised comparisons of *N. gonorrhoeae* strains (Moodley *et al* 2006; Sethi *et al*., 2013).

2.4 *Trichomonas vaginalis*: a sexually transmitted protozoan

Protozoa generally account for the highest number of STI worldwide (Moldwin, 1992). These organisms can be eradicated in the infected individual but its high asymptomatic carriage rates have a significant influence on its continued transmission (Moldwin, 1992). *Trichomonas vaginalis* is a flagellated protozoan that causes trichomoniasis, a common but overlooked sexually transmitted human infection (Carlton *et al*., 2007). It account for over 170 million cases of trichomoniasis annually worldwide (Carlton *et al*., 2007). The best hope for eradication of these organisms lies in increasing the index of suspicion in high-risk populations and the careful evaluation and treatment of sexual partners of infected persons (Moldwin, 1992).

2.4.1 History of *T. vaginalis*

Trichomonas tenax was the first trichomonad species to be described (Campbell, 2001). It was isolated from a dental plaque by Miller in 1773 (Campbell, 2001). It is now an established commensal of the human oral cavity and has been linked with gingivitis (Athari *et al*., 2007). Donne described the first *T. vaginalis* isolated from the genital secretion of a woman in 1836 (Sood and Kapil, 2008; Harp and Chowdhury, 2011). Although isolated in a female, Donne pointed out that it can be found in the genital secretions of females and males alike (Campbell, 2001; Valadkhani *et al*., 2008). He described it as a morphological blend between two organisms *Tricodes* and *Monas,* thus he named the protozoa *Trico-monas* and assigned it to the epithet *vaginalis* (Campbell, 2001).

2.4.2 Classification of *T. vaginalis*

Trichomonas vaginalis is a member of the parabasilid lineage of microaerophilic eukaryotes (Carlton *et al*., 2007). It is considered to be one of the earliest branching eukaryotic lineages (Carlton *et al*., 2007). Parabasalia are characterised by the presence of a parabasal body: Golgi associated with kinetosomes; an axostyle and a undulating membrane (Schwebke and Burgess, 2004). The *Diplomonads* also belong to the *Excavata* and is most closely related to the *Parabasalids* (Gerbod *et al*., 2004; Schwebke and Burgess, 2004). *Giardia lamblia* is a famous species of the *Diplomonads* (Gerbod *et al*., 2004). It is commonly known as ‘‘Beaver Fever’’, which causes severe diarrhoea (Gerbod *et al*., 2004; Schwebke and Burgess, 2004).

Table 2.2: **Scientific classification of *T. vaginalis* (Schwebke and Burgess, 2004)**

Taxonomic rank	Scientific name
Domain	*Eukaryota*
Phylum	*Zoomastigina*
Class	*Parasabalia*
Order	*Trichomonadia*
Family	*Trichomonadidae*
Genus	*Trichomonas*
Species	*T. vaginalis*
Binomial name	***Trichomanas vaginalis***

Members of the order *Trichomonadida* has four to six flagella, free or attached to an undulating membrane; no true cysts (Schwebke and Burgess, 2004). The genus *Trichomonas* contains organisms with four free flagella; one recurrent, along the outer margin of the undulating membrane; a costa at the base of the undulating membrane and an axostyle extending through the cell (Schwebke and Burgess, 2004).

2.4.3 General characteristics of *T. vaginalis*

Trichomonas vaginalis is an anaerobic, parasitic, flagellated protozoan (Dunne *et al.,* 2003). It is a protozoan with an oval or pear-shape and the size of about two times the size of a polymorphonuclear leukocyte (Dunne *et al.,* 2003). It measures from 12 to 24 µm in length and from 6 to 18 µm in width (Dunne *et al.,* 2003). The size is dependent on the environment's physicochemical conditions (Benchimol, 2004; Ali and Nozaki, 2007). In favourable conditions the shape is uniform, being pear shaped or oval with visible flagella (Ali and Nozaki, 2007). However, during unfavorable growth conditions, the trichomonads internalise its flagella to form a round shape (Benchimol, 2004; Ryan *et al*., 2010). *Trichomonas vaginalis* is highly motile; it drives itself with four whip-like flagella (Benchimol, 2004; Ryan *et al*., 2010).

Trichomonas vaginalis has a visible nucleus situated anteriorly (Ryan *et al*., 2010). It lacks mitochondria and peroxisomes but contain unusual organelles called hydrogenosomes (Carlton *et al*., 2007). It has a cell membrane but lacks a cell wall and has a the mouth-like

structure (cytosome) which is used to derive nutrients from the environment (Ryan *et al*., 2010). *Trichomonas vaginalis* reproduces every 8 to 12 hours under optimal conditions by mitotic division of the trichomonads occurring by longitudinal binary fission (Cudmore *et al*., 2004; Sutcliffe *et al*., 2006).

The draft genome sequence of *T. vaginalis* published in 2007 is a highly fragmented assembly and at least 65% of it is repetitive (Carlton *et al*., 2007; Conrad *et al*., 2013). The sequenced genome contains approximately 60 000 predicted protein-coding genes, 1100 ribosomal RNA genes and at least 14 390 viral or transposable element open reading frames (ORFs) (Conrad *et al*., 2013).

2.4.4 Virulence factors and pathogenesis of *T. vaginalis*

As an extracellular parasite, *T. vaginalis* colonises the mucosal surfaces of the human genitourinary tract without direct invasion (Goodman *et al*., 2011). Successful colonisation of the mucous membranes and the maintenance of infection are favoured by the existence of many virulence mechanisms (Figueroa-Angulo *et al*., 2012). These mechanisms include binding and degradation of mucosal and extracellular matrix (ECM) components and the adherence to and cytotoxicity of vaginal epithelial cells (VECs) and cervical (HeLa) and prostatic cells (DU145) (Figueroa-Angulo *et al*., 2012).

After exposure, *T. vaginalis* initially adheres to and infects the vagina and then moves to the uterus and oviduct, the duration of infectiousness is usually 1 to 120 days (Bowden and Garnett, 2000; Benchimol *et al*., 2008). To establish and maintain a chronic infection in the human urogenital tract, *T. vaginalis* uses surface proteins (adhsins), proteins in the cytoskeleton, the receptor proteins (laminin, fibronectin, and collagen) and signal transduction to coordinate cytoadherence (Figueroa-Angulo *et al*., 2012). After attachment to host cells, *T. vaginalis* uses phagocytosis and endocytosis strategies to evade the host immune system (Lehker and Alderete, 2000; Meza-Cervantez *et al*., 2011). Phagocytosis destroys vaginal microorganisms (bacteria, viruses, yeast, etc.) and host cells (VECs, erythrocytes, spermatozoa, etc.) (Pereira-Neves and Benchimol, 2007; Benchimol *et al*., 2008). Endocytosis of host proteins degrades the complement proteins and immunoglobulins (Figueroa-Angulo *et al*., 2012). Surface molecular mimicry and masking with host proteins are other mechanisms used to evade the host immune system (Figueroa-Angulo *et al*., 2012).

2.4.5 Immune response to *T. vaginalis* infections

In males *T. vaginalis* infection is self-limiting (Pattullo *et al*., 2009) while in females the cervico-vaginal cells protect the genital tract by recognising microorganisms and reacting with innate immune and inflammatory responses in an effort to maintain a normal healthy microbiome (Wira *et al*., 2005). This process involves co-operation between the ectocervical epithelium, vaginal mucosa and genital tract microflora, mediated by Toll-like receptors (TLRs) and an array of cytokines, chemokines and innate immune factors (Thurman and Doncel, 2011). The innate and adaptive immune cell produces interleukin-22 (IL-22) cytokines (Makinde *et al*., 2013), which are expressed on the epithelial cells and it functions as barrier and also promotes epithelial homeostasis (Makinde *et al*., 2013). These cells serve as the first line of defence against *T. vaginalis* infections.

Leukocytes produce a number of biochemical molecules: IL-8, leukotrienes reactive nitrogen intermediates, macrophage and inflammatory protein-3α (Crouch *et al*., 2001; Lubick and Burgess, 2004; Gomez *et al*., 2010). Leukocytes stimulate nitric oxide synthase (Inos), site-specific immunoglobulin G (lgG) and A (lgA) and promote transmigration of neutrophils across the endothelium (Crouch *et al*., 2001; Lubick and Burgess, 2004; Gomez *et al*., 2010). The assault of the *T. vaginalis* membranes results in bursting, which lead to the disintegration and discharge of its organelles (Lubick and Burgess, 2004; Gomez *et al*., 2010).

2.4.6 Clinical manifestation of *T. vaginalis* infections

The clinical manifestations of trichomoniasis are non-specific and infection is very often asymptomatic (Hobbs and Seña, 2013). *Trichomonas vaginalis* affects both men and women although the clinical manifestation differs (Swygard *et al*., 2004). The clinical presentations of trichomoniasis in women range from asymptomatic to severe vaginitis characterised by diffuse, malodorous discharge or urethritis characterised by dysuria (Hobbs and Seña, 2013). Symptomatic *T. vaginalis* infection may increase the risk of adverse pregnancy outcomes including preterm delivery and delivery of a low-birth-weight infant (Hoots *et al*., 2013).

Cervicitis caused by *T. vaginalis* is characterised by petechial haemorrhages on the ectocervix ‘‘strawberry cervix’’ may distinguish trichomoniasis from other causes of cervicitis (Hobbs and Seña, 2013). In men, *T. vaginalis* can cause asymptomatic infections, nongonococcal urethritis characterised by urethral discharge and dysuria (Hoots *et al*., 2013). Infection may result in complications, such as prostatitis (Hoots *et al*., 2013).

2.4.7 Diagnosis of *T. vaginalis*

Disease history; physical examination based on the odour, quality and quantity of vaginal discharge; the vaginal pH; and possible presence of cervical friability are suggestive of *T. vaginalis* infection (Van Der Pol *et al*., 2006). However, the absence of these clinical signs is not sufficient evidence to rule out infection (Van Der Pol, 2012). Accordingly, to enhance the sensitivity and specificity of diagnosis, laboratory methods are required (WHO, 2013).

There are four classes of laboratory diagnostic assays for detecting *T. vaginalis*, this include; antigen detection, culture, nucleic acid amplification tests (NAATs) and wet preparation microscopy (WHO, 2013). Wet mount microscopy involves the visualisation of *T. vaginalis*; characteristic size, morphology and characteristic trichomonad movements (tumbling) seen in the physiological saline preparation of genital secretions or urine (Garber, 2005). This method must be performed and interpreted within 10 minutes for optimal results and has highest sensitivity in symptomatic women (WHO, 2013). The sensitivity of microscopy varies from 50% to 66% with a specificity of 100% (Pattullo *et al*., 2009).

The broth culture method is the most common method used for the diagnosis of trichomoniasis because it is simple to interpret and requires less than 300 trichomonads per ml to initiate growth (Crucitti *et al*., 2003; Soper, 2004). Culture is usually performed using modified Diamond's medium or the commercial InPouch TV culture system (BioMed Diagnostics, USA) (Soper, 2004; WHO, 2013). The Diamond's media will successfully identify up to 95% of the *T. vaginalis* infections, making it a better technique than the wet mount microscopy, because it achieves both a high degree of sensitivity 85% to 95% and specificity >95% (Cudmore *et al*., 2004). The disadvantage of this method is the delay in making a definitive diagnosis, because of the incubation period of two to seven days required to identify *T. vaginalis* in culture (Hobbs and Seña, 2013).

Various techniques including agglutination, complement fixation, indirect hemagglutination, gel diffusion, fluorescent antibody and enzyme-linked immunosorbent assay (ELISA) have been used to demonstrate the presence of anti-trichomonal antibodies (Garber, 2005). Xeno-Strip-Tv; (Xenotope Diagnostics, Inc., San Antonio, Texas) is a point-of care, lateral-flow test strip device that detects *T. vaginalis* membrane proteins (Pattullo *et al* 2009). Its sensitivity (85% to 90%) and specificity (100%) are similar to those of culture (Pattullo, *et al* 2009).

2.4.8 Treatment of *T. vaginalis*

Metronidazole and other 5-nitroimidazoles (tinidazole, ornidazole and secnidazole) have become the treatment of choice for *T. vaginalis* infections (Gehring and Efferth, 2009; Goldman *et al*., 2009; Wright *et al*., 2010). The effectiveness of the drug is high, with curing rates of approximately 85% to 95%; re-infections can be prevented through simultaneous treatment of sexual partners (Frenkl and Potts, 2008). The efficacy of tinidazole equals or is superior to that of metronidazole because of it plasma elimination half-life (Gehring and Efferth, 2009; Johnson, 2009). Unlike metronidazole, tinidazole is expensive and thus cannot be used routinely in developing countries like South Africa (Kissinger *et al*., 2008).

2.5 *Chlamydia trachomatis*: the silent epidemic

Chlamydia trachomatis is a bacterial STI common among all socioeconomic group and it disproportionately affects young people under the age of 25 years (Lorimer and McDaid, 2013). *Chlamydia* has been referred to as a "silent epidemic" due to its largely asymptomatic course, which provides infected people with no or few visible signs with which to seek health care (Lorimer and McDaid, 2013). Chlamydial infections are readily treated but have potentially serious complications for untreated disease (Pourat *et al*., 2011). Unrecognized and untreated *C. trachomatis* infection can contribute to the spread of infection in a community (Walsh *et al*., 2000; Pourat *et al*., 2011).

The CDC has recommended *C. trachomatis* screening of sexually active young women under the age of 20 years and older women with risk factors for infection over twenty years ago (Walsh *et al*., 2000; Pourat *et al*., 2011). This recommendation is as a result of the asymptomatic nature and the untreated diseases outcome (Pourat *et al*., 2011). A screening program is a systematic and organized provision of regular *Chlamydia* testing to reach a defined population (ECDC, 2013b). A screening programme can either be random or proactive (Lorimer and McDaid, 2013).

A survey by the European Centre for Disease Prevention and Control (ECDC) in 2008 reported no organised *Chlamydia* screening activity across Europe (ECDC, 2013a). However, nine countries, including France, Ireland and the Netherlands, had plans to introduce screening programmes in the future (ECDC, 2013a). A subsequent survey in 2012 to assess current and planned *Chlamydia* control activities in 33 countries across Europe by Low *et al*. (2013) identified only nine countries with plans to introduce a screening programme. Norway

is the only country exploring and planning a proactive screening approach according to this survey (Low *et al*., 2013). *Chlamydia trachomatis* is likely to continue to constitute a silent epidemic. This is because, despite the long-standing recommendations, current levels of routine *Chlamydia* screening are low (Walsh *et al*., 2000; Low *et al*., 2013). Hence, health gain and financial benefits can only be achieved by active case finding and treatment of *C. trachomatis* infection, which can be done on an individual level or nationwide in a screening programme (Rours *et al*., 2008).

2.5.1 History of *Chlamydia trachomatis*

Chlamydia trachomatis was discovered in 1907 by Halberstaedter and Von Prowazek who observed it in conjunctival scrapings from an experimentally infected orang-utan (primate) (Budai, 2007). The word chlamys is Greek for "cloak draped around the shoulder" (Lewis and Maruma, 2009). Chlamys describes how the intra-cytoplasmic inclusions caused by the bacterium are "draped" around the infected cell's nucleus (Stephens, 2003). Trachomatis is a Greek word and it means rough or harsh (Budai, 2007). *Chlamydia* was not recognized as a STI until it was isolated in 1957 (Stephens, 2003).

Over the last hundred years the detection and study of intracellular pathogens, including *Chlamydiae*, passed through an enormous evolution (Budai, 2007). The laboratory services for the diagnosis of *C. trachomatis* became feasible only in the early 1980s (Chernesky *et al*., 2005). During this period cell culture systems were developed for the inoculation of clinical specimens (Chernesky *et al*., 2005). This innovation complemented cytological methods and moved the diagnostic field forward (Howell *et al*., 1998).

2.5.2 Classification of *Chlamydia trachomatis*

Chlamydiae are an obligate intracellular bacterium that was first thought to be protozoa and later a virus, but it became clear that *Chlamydiae* had all the important features of bacteria (Stephens, 2003). *Chlamydiae* are placed in the order, *Chlamydiales*, with one family, *Chlamydiaceae* (Stephens, 2003). *Chlamydia trachomatis* is a member of the family *Chlamydiaceae* which contains two genera *Chlamydia* and *Chlamydophila* (Garrity *et al*., 2004). Members of the two genera are separated based on apparent differential clustering of the 16S rRNA gene (Garrity *et al*., 2004).

This bacterial family has representatives extensively distributed in nature (Stephens *et al.,* 1998). The most important human species are *Chlamydia trachomatis*, *Chlamydopila pneumoniae* and *Chlamydophila psittaci* (Darville, 2005). The distinguishing characteristics between these species include; the host range, clinical expression and the staining characteristics (due to glycogen inclusions) (Darville, 2005 and Stephens *et al*., 2009).

Table 2.3: **Scientific classification of *C. trachomatis* (Garrity *et al*., 2004; Euzéby, 2012)**

Taxonomic rank	Scientific name
Domain	*Bacteria*
Phylum	*Chlamydiae*
Class	*Chlamydiae*
Order	*Chlamydiales*
Family	*Chlamydiaceae*
Genus	*Chlamydia*
Species	*C. trachomatis*
Binomial name	***Chlamydia trachomatis***

Chlamydia trachomatis is divided into subgroups based on antigenic variation in the major outer membrane proteins (MOMP) (19 serovars) and on clinical expression (biovars) (Joubert and Sturm, 2011). Analysis of clinical disease and the plasticity zone suggests that *C. trachomatis* species should be divided into three biovars (Carlson *et al*., 2005; Joubert and Sturm, 2011), the trachoma biovar, the genital biovar and the lymphogranuloma venereum (LGV) biovar (Joubert and Sturm, 2011). Seventy percent of the non-LGV STIs are due to serovars D and E (Martin-Iguacel *et al*., 2010).

2.5.3 General characteristics of *Chlamydia trachomatis*

Chlamydia trachomatis is a non-motile, Gram-negative obligate intracellular bacterium (Somani *et al*., 2000). It has double cell wall but lacks a peptidoglycan layer (Hogan *et al.,* 2004). Similar to other Gram-negative bacteria it has lipopolysaccharide and has the ability to produce its own proteins and nucleic acids (Demars *et al*., 2007). *Chlamydia trachomatis* is slow growing and hardly exchange DNA with other related *Chlamydiae* (Demars *et al*., 2007). The organism is also extremely temperature sensitive and must be refrigerated at 4^{o}C as soon as a sample is obtained (Lewis and Maruma, 2009). It lacks the ability to produce endogenous adenosine triphosphate (ATP) hence *C. trachomatis* is referred to as energy parasite (Carlson *et al*., 2005). The central metabolic pathway of *C. trachomatis* is aerobic, with glutamate as the primary carbon source supplemented by glucose (Stephens *et al*., 1998).

Chlamydia trachomatis is characterised by a unique two phase life cycle, that alternate between the elementary body (EB) and the reticulate body (RB) (Hogan *et al.,* 2004). The EB is a metabolically inactive developmental form that acts like fungal spores and is responsible for transmission of infection, while the RB is a metabolically active developmental form (Manavi, 2006).

The genomes of representative biovars of *C. trachomatis* have been sequenced and subsequent genome comparison has revealed important information on its evolution (Albrecht *et al*., 2010). All four bivars has a small and highly conserved genome of approximately 1 000 kb size (1 042 519 bp) and contains both RNA and DNA (Carlson *et al*., 2005; Albrecht *et al*., 2010; Wang *et al*., 2011). The sequenced genomes contain 58.7% A+T nucleotide, 7 493 bp plasmid DNA, 894 protein coding sequence (CDS) and a difference of 5 000 bp between the biovars (Stephens *et al.,* 1998; Albrecht *et al*., 2010; Wang *et al*., 2011). The cryptic plasmid carries noncoding RNAs and eight conserved open reading frames (ORFs) designated *pgp*1 to 8 (Song *et al*., 2013). *Chlamydia trachomatis* genome shows high coding density of 90% demonstrating a highly optimised usage of the coding capacity of its genomes (Albrecht *et al*., 2010). The Swedish new variant of *C. trachomatis* (nvCT) carries a plasmid with a 377 bp deletion within the CDS1 and a 44 bp duplication immediately upstream of both CDS2 and CDS3 (Unemo *et al*., 2010).

2.5.4 Virulence factors and pathogenesis of *C. trachomatis*

A number of virulence factors have been identified in *C. trachomatis* and some of these factors are common across the genus level (Byrne, 2010). These virulence factors include; adhesins, the polymorphic outer membrane auto-transporter family of proteins, the putative large cytotoxin, type III secretion system (TTSS) effectors, chlamydial LPS, stress response proteins and other regulatory factors produced by the cryptic plasmid (Wehrl *et al*., 2004; Byrne, 2010).

Chlamydia trachomatis has a complex mechanism for host cell entry and attachment (Moelleken and Hegeman, 2008). The putative cytotoxic activity contributes to it disease pathology (Belland *et al*., 2001). The *C. trachomatis* genome encodes proteins with significant homology to the large clostridial toxin B protein (Belland *et al*., 2001; Byrne, 2010). This cytotoxin produces morphological and cytoskeletal damage to the epithelial cells (Belland *et al*., 2001; Byrne, 2010). The LGV strains lack the toxin gene (Byrne, 2010). The TTSSs are complex arrangements of structures that promote delivery of the pathogen effector proteins after contact with the host cell (Cornelis and Van Gijsegem, 2000). This effector protein accelerates the infection process (Byrne, 2010).

Chlamydia trachomatis infection is sexually acquired through both penetrative sexual inter-course and oral sex, incubation period for the infection is 7 to 21 days (Horner, 2005; Manavi, 2006). Essentially, the chlamydial life cycle constitutes its pathogenic process (Manavi, 2006). During invasion of host cells, the EB binds, enters a suitable cell and induces its own endocytosis, matures to form a RB, which undergoes binary fission (Manavi, 2006; Miyairi *et al.,* 2006). After the incubation period and multiple divisions by binary fission, the RB differentiates into the EB developmental stage and the infectious EB are released to initiate new rounds of infection through exocytosis (Stephens *et al.,* 1998; Miyair *et al.,* 2006). Inflammatory pathology, cellular proliferation, scarring, tissue remodelling and damage that are evidence during chronic persistent infection are as a result of autoimmunity (over production of inflammatory mediators by host cell) (Horner, 2005; Gottlieb *et al*., 2010).

2.5.5 Immune response to *C. trachomatis* infections

Diseases caused by *Chlamydia* are thought to be mediated by immunopathology (Stephens, 2003). *Chlamydia* disease is mediated by antigen-dependent delayed-type hypersensitivity or autoimmunity (Stephens, 2003). Two paradigms have been described on how the host

immune systems respond to chlamydial infection namely; the cellular paradigm and the immunological paradigm (Gottlieb *et al*., 2010). The evidence related to these paradigms has come from *in vitro*, mouse and primate model data (Gottlieb *et al*., 2010).

According to the immunological paradigm, infection is characterised by mucosal infiltration with neutrophils and modest numbers of monocytes (Morrison and Morrison, 2000). Neutrophils are often recruited in large numbers to the site of infection and are capable of killing accessible EB (Darville and Hiltke, 2010). Later, T-cells accumulate at the site of infection and play a vital role in clearing the infection (Darville and Hiltke, 2010). Generally, the chlamydial-specific adaptive T-cell responses that develop over time to help clear infection are thought to induce collateral tissue damage if it fails to clear infection (Gottlieb *et al*., 2010).

Under the cellular paradigm, the host response is initiated and sustained by epithelial cells that are the primary targets of chlamydial infection (Stephens, 2003). As such, infected host epithelial cells act as first defence, initiating and propagating immune responses (Darville and Hiltke, 2010). It secretes chemokines that recruit inflammatory leukocytes to the site of the infection and cytokines that induce and augment the cellular inflammatory response (Darville and Hiltke, 2010; Gottlieb *et al*., 2010). If infection is not cleared by the secreted chemokines and cytokines, the inflammatory mediators from both infected host cells and the infiltrating immune cells induce direct damage to the tissues (Phillips and Shor, 2001; Darville and Hiltke, 2010).

2.5.6 Clinical manifestations of *C. trachomatis* infection

Chlamydial infection can cause inflammation of the cervix and urethra in women and in men respectively (Bjartling *et al*., 2009). Asymptomatic carriage of *C. trachomatis* is as high as 70% of women and 50% of men and thus remains undetected (Van de Laar and Morre, 2007; Brunhama and Rappuolib, 2013). *Chlamydia trachomatis* originating from the genital tract is associated with reactive arthritis, which develops in 1% to 3% of patients after genital chlamydial infection (Hogan *et al.,* 2004).

Cervicitis due to *C. trachomatis* can be asymptomatic or may present with vaginal discharge (Horner, 2005; Brunhama and Rappuolib, 2013). Ascending infection of the female upper genital tract can result from cervicitis eventually causing PID (Hogan *et al.,* 2004). Pelvic inflammatory disease causes salpingitis, which in turn leads to fibrosis and scarring of the

fallopian tubes, ectopic pregnancy and tubal infertility (Hogan *et al.,* 2004; Brunhama and Rappuolib, 2013). Endometritis is frequently associated with this and may produce irregular uterine bleeding (Rogstad, 2008).

Chlamydia trachomatis is the main cause of NGU in men (Brunhama and Rappuolib, 2013). Urethritis can be complicated by severe epididymitis in young men (Bébéar and De Barbeyrac, 2009). The symptoms after 7 to 21 days of infection include dysuria and a moderate clear or whitish urethral discharge (Peipert, 2003). Reiter's syndrome (urethritis, conjunctivitis, arthritis and mucocutaneous lesions) or reactive arthritis has also been linked with genital *C. trachomatis* infections in men (Hicks, 2008).

Chlamydia trachomatis is the most frequent identifiable cause of neonatal conjunctivitis in many countries (De Vries *et al*., 2008). Infants of mothers with chlamydial infections can be infected at delivery (vertical transmission) (Rours *et al*., 2008). The untreated infection acquired at birth can persist for months or years (Bébéar and De Barbeyrac, 2009). In adults, conjunctivitis may occur by auto-inoculation of *C. trachomatis* from the genital tract (Paavonen and Eggert-Kruse, 1999).

2.5.7 Diagnosis of *C. trachomatis*

The methods used for the laboratory diagnosis of *C. trachomatis* infection are rapidly developing (Horner, 2005). Several methods are now available, all of which have high specificity and variable sensitivity (Horner, 2005). Cell culture, direct fluorescence antibody (DFA) testing, enzyme immunoassay (EIA) and nucleic acid amplification tests are now readily available (Chernesky, 2005).

The choice of specimen required for the detection of *C. trachomatis* depends on the clinical picture and the laboratory technique available (Bébéar and De Barbeyrac, 2009). When taking specimens one should bear in mind that *Chlamydiae* are obligate intracellular pathogens and that specimen should therefore have epithelial cells from the involved sites rather than exudates (Chernesky, 2005).

The microscopy method has a sensitivity that exceeds 60% when diagnosing neonatal conjunctivitis (Chernesky, 2005). This method relies on the microscopic examination of cell scrapings stained with iodine (Giemsa staining), for the presence of typical intra-cytoplasmic inclusion bodies (Chernesky, 2005). Cell culture has almost 100% specificity for the

detection of *C. trachomatis*; it is the preferred method for antibiotic susceptibility testing (Warford *et al.,* 1999; Bébéar and De Barbeyrac, 2009). Despite cell culture being the gold standard for the detection of *C. trachomatis* it is not recommended for routine use, because of its lack of sensitivity, its technical complexity, long turn-around time and specimen transport and storage requirements (Essig, 2007).

The DFA is rapid to perform and specific, but it is subjective and not suitable for a large number of specimens (Black, 1997). This method is highly specific (99%) compared to culture, but has a lower sensitivity of 75% to 85% (Svensson *et al*., 1991). The EIA test is a rapid method for detecting *C. trachomatis* (Mahilum *et al*., 2007). This method uses either monoclonal or polyclonal antibodies to detect chlamydial LPS (Chernesky, 2005; Bébéar and De Barbeyrac, 2009). The sensitivity profiles of the commercially available *C. trachomatis* EIAs ranges from 65% to 75% compared with NAA assays (Leber *et al*., 2006; Michel *et al*., 2006). This method offers the advantage of 'point-of-care' test for patients (Mahilum *et al*., 2007). Enzyme immunoassay tests are not suitable for non-invasive specimens and these tests are not recommended in clinical settings (Michel *et al*., 2006).

The first molecular DNA test for *C. trachomatis* was a nucleic acid hybridisation test (NAH) (Bébéar and De Barbeyrac, 2009). A commercially available NAH test is PACE 2 (Gen-Probe Inc, USA). This probe test is relatively specific and provides sensitivity similar to that of the cell culture method (Schachter *et al*., 2005). Endocervical or urethral swabs can be used with PACE 2, but it is not recommended for use with urine or self-collected vaginal swabs (Schachter *et al*., 2005). Second generation NAH test is based on the detection of an amplified signal (Leber *et al*., 2006). The sensitivity of the hybrid Capture II test (Digene Corporation, USA) is significantly higher than that of the PACE 2 test and is comparable to that of PCR (Leber *et al*., 2006).

Nucleic acid amplification techniques are more sensitive (90% to 95%) and specific (99% to 100%) compared with cell culture and EIA test because it amplifies specific DNA or RNA nucleic acid sequences unique to *C. trachomatis* in clinical specimens (Van Dyck *et al*., 2001; Currie *et al*., 2004; Schachter *et al*., 2006). These methods can be used to detect *C. trachomatis* from a large range of sample types, including non-invasive specimen, such as vaginal swabs and first-void urine (FVU) (Leber *et al*., 2006).

The nucleic acid sequences unique to *C. trachomatis* that is commonly targeted by commercially available assays include; cryptic plasmid, ribosomal RNA and *omp*A, these genes are present in multiple copies within the EB (Chernesky, 2005). The Swedish new variant of *C. trachomatis* (Ripa and Nilsson, 2006), has a 377-bp deletion in the cryptic plasmid (Ripa and Nilsson, 2006). This variant can only be detected by an assay targeting the rRNA or the *omp*A genes (Bébéar and De Barbeyrac, 2009).

2.5.8 Treatment of *C. trachomatis*

The management of chlamydial infection is based on treatment of the patient and his or her infected sexual partner(s) (Manavi, 2006). Chlamydia infection is easily treated as *C. trachomatis* is susceptible to macrolides, quinolones and tetracyclines (Manavi, 2006). The recommended regimens are: azithromycin single dose of 1 g, erythromycin 500 mg twice daily for 14 days and doxycycline 100 mg twice daily for seven days (Manavi, 2006; Kalwij *et al*., 2010). Pregnant women erythromycin: 500 mg four times daily for seven days or 500 mg twice daily for 14 days (Kalwij *et al*., 2010).

2.6 M*ycoplasma genitalium:* an elusive 'minimal genome' bacterium

The search for the 'smallest autonomous self-replicating entity' started in the 1960s when *Mollicutes* were recognised as the smallest microorganisms on Earth (Glass *et al*., 2006). With the emergence of molecular biology, the object of the search for the smallest organism shifted towards the organism with the smallest genome (Acevedo-Rocha *et al*., 2013). *Mycoplasma genitalium* has a genome size of 580 kb and requires only 265 essential protein CDS for survival (Acevedo-Rocha *et al*., 2013). This makes *M. genitalium* a model organism to study the elusive 'minimal genome' (Acevedo-Rocha *et al*., 2013). A minimal genome is the minimal set of genes required by an organism to support cellular life in an ideal environment (Acevedo-Rocha *et al*., 2013).

The central undertaking in synthetic biology is the search for the 'minimal genome' (Tavassoli, 2010). The goal therefore, is to engineer cells for useful applications, while contributing to our understanding of the origin of life on Earth (De Lorenzo and Danchin, 2008). This goal can be achieved by the use of minimal genome as a scaffold onto which genes can be added, followed by its transplantation into a chassis (Tavassoli, 2010). Finally, specialised genes can be added to the chassis to create 'turbo cells' for applications in the

fields of energy production, health and the environment (Vickers *et al*., 2010; Acevedo-Rocha *et al*., 2013).

2.6.1 History of *M. genitalium*

In 1937, Dienes and Edsall isolated and reported the first mycoplasma that was pathologic to human from a Bartholin's gland abscess (Waites *et al*., 2005). Mycoplamas was called pleuropneumonia-like organisms at that time, because the microbe had been shown to cause bovine pleuropneumonia (Waites *et al*., 2005; Razin and Hayflick, 2010). The term mycoplasma is derived from the Greek word mykes meaning fungus and plasma-form (Waites *et al*., 2005). Mykes was used to describe the pleuropneumonia-like organisms in the 1950s (Waites *et al*., 2005).

Mycoplasma genitalium was first isolated in 1980 by culture of urethral discharge from two men with NGU (Leung *et al*., 2006). The events leading to this discovery includes, the observation that some men responded to tetracycline therapy despite the failure to detect bacteria in the urethra during studies of acute NGU in the 1970s (Taylor-Robinson and Jensen, 2011). The dark-field microscopy of fresh urethral smears from a few men with NGU, some of whom were infected by chlamydiae and ureaplasmas, sometimes revealed motile spiral forms (Taylor-Robinson and Jensen, 2011). These were not *Treponema pallidum* but to some extent resembled spiroplasmas of plants and insects (Taylor-Robinson and Jensen, 2011). Further research led to the isolation of *M. genitalium* from the urethral swab of two men with NGU attending a STD clinic at St. Mary's Hospital, Paddington, London, United Kingdom in 1980 (Manhart and Kay, 2010; Taylor-Robinson and Jensen, 2011). These isolates were subsequently named *M. genitalium* and designated G-37 and M-30 (Le Roux and Hoosen, 2010). The G-37 genome was fully sequenced in 1995 and it's now an American Type Culture Collection (ATCC 33530) strain (Le Roux and Hoosen, 2010).

2.6.2 Classification of *M. genitalium*

Mycoplasmas belong to the class *Mollicutes* (bacteria without a cell wall) (Svenstrup *et al*., 2006; Jensen, 2006). *Mollicutes* contains four orders, five families, eight genera and more than 200 known species that have been detected in humans, vertebrate animals, arthropods, and plants (Waites *et al*., 2005). The order *Mycoplasmatales* with a single family *Mycoplasmataceae* (Table 2.4) is the most important from a human perspective (Jensen, 2006; Euzéby, 2012). *Mycoplasmatacea* contain two genera, namely *Mycoplasma* and

Ureaplasma (Jensen, 2006; Euzéby, 2012). The genus *Mycoplasma* contains over one hundred species and several of these species are considered commensals (Waites *et al*., 2005). Four species in the genus *Mycoplasma* are proven human pathogens: *M. pneumoniae*, *M. genitalium*, *M. hominis* and *M. fermentans* (Waites *et al*., 2005).

Table 2.4: Scientific classification of *M. genitalium* (Garrity *et al*., 2004; Euzéby, 2012)

Taxonomic rank	Scientific name
Domain	*Bacteria*
Phylum	*Firmicutes*
Class	*Mollicutes*
Order	*Mycoplasmatales*
Family	*Mycoplasmataceae*
Genus	*Mycoplasma*
Species	*M. buccale, M. fermentans, M. genitalium,* etc
Binomial name	***Mycoplasma genitalium***

2.6.3 General characteristics of *M. genitalium*

Mycoplasmas are the smallest prokaryotes capable of self-replication and these bacteria have high mutation rates (Iversion-Cabral *et al*., 2006; Jensen, 2006). Mycoplasmas are thought to have evolved from Gram-positive bacteria most closely related to the clostridia by degenerative evolution (Le Roux and Hoosen, 2010). *Mycoplasma genitalium* have the smallest genome of all *Mollicutes* (Iversion-Cabral *et al*., 2006; Le Roux and Hoosen, 2010). It has a flask-shaped morphology and terminal attachment organelles (Cazanave *et al.,* 2012). *Mycoplasma genitalium* are pleomorphic and cannot be Gram stained and identified by optical microscopy (Deguchi and Maeda, 2002; Cazanave *et al.,* 2012). *Mycoplasma genitalium* has a guanine-plus-cytosine (G+C) content of 32% and it metabolises glucose but not arginine or urea (Taylor-Robinson and Jensen, 2011). *Mycoplasma genitalium* is characterised by the requirement for complex growth media and utilisation of UGA as a tryptophan codon (Svenstrup *et al*., 2006).

Mycoplasma genitalium exhibits gliding motility, reproduces by binary fission and has a cytoskeleton-like structures that function in modulating cell shape (Razin and Hayflicks, 2010; Taylor-Robinson and Jensen, 2011). It grows very slowly and produces a "fried-egg"-like colony in an atmosphere of nitrogen with 5% CO_2 (Deguchi and Maeda, 2002; Razin and Hayflick, 2010). *Mycoplasma genitalium* adheres to glass and plastic surfaces and to various human and animal cells mainly by an *M. genitalium* attachment (adhesin) protein (Taylor-Robinson and Jensen, 2011).

2.6.4 Virulence factors and pathogenesis of *M. genitalium*

Mycoplasmas are surface parasites of the mucous membrane cells (Jensen, 2006). It hardly causes serious infection nor kills its host; rather infections tend to follow a chronic path (Jensen, 2006). *Mycoplasma genitalium* primarily infects the urogenital track (Jensen, 2006). It can attach to different cell types, including erythrocytes (Morrison-Plummer *et al*., 1987), Vero cells (Jensen *et al*., 1994), Fallopian tube cells (Collier *et al*., 1990), respiratory cells (Baseman *et al*., 1996) and spermatozoa (Svenstrup *et al*., 2003; Le Roux and Hoosen, 2010).

The main virulence mechanism in *M. genitalium* is adhesion to host cells (Cazanave *et al.,* 2012). Other mechanisms include motility, invasion of host cells and toxin production (Taylor-Robinson and Jensen, 2011). The adhesion of *M. genitalium* to host cell involves very complex processes that utilises several proteins (Taylor-Robinson and Jensen, 2011). The main adhesion protein of *M. genitalium* is the 140 kDa *M. genitalium* adhesion protein (MgPa), encoded by the *mgp*B gene (Cazanave *et al.,* 2012). These proteins allow the bacterium to adhere to various materials and cells including epithelial cells and spermatozoa (Le Roux and Hoosen, 2010; Cazanave *et al.,* 2012). *Mycoplasma genitalium* exhibits gliding motility (Hatchel and Balish, 2008; Razin and Hayflick, 2010).

Gliding is an important virulence factor, as it aids *M. genitalium* in penetrating the mucous layer covering mucosal epithelial cells and enabling it to attach to cells after the onset of infection (Taylor-Robinson and Jensen, 2011). Glyceraldehyde-3-phosphate dehydrogenase plays a role in binding the organism to mucin on host cells (Alvarez *et al*., 2003). Proteins P140 and P110 of the MgPa are used in order to escape the host immune attack (Le Roux and Hoosen, 2010). This protein undergoes phase and antigenic variation by altering the entire genetic sequence of the MgPa with subsequent generation of variants (*Mgp*A and *Mgp*C) that

are not recognised by the host immune system on subsequent encounters (Jensen, 2006; Ueno *et al*., 2008; Burgos and Totten, 2014).

Intracellular localisation contributes to the chronicity of infections (Waites and Talkington, 2004). *Mycoplasma genitalium* produces the MG-186 enzyme, which has the ability to degrade host cell nucleic acids as a source of nucleotide precursors for growth and for pathogenic processes (Taylor-Robinson and Jensen, 2011).

2.6.5 Immune response to *Mycoplasma genitalium* infections

The host immune system (monocytes/macrophages and lymphocytes) responds to bacterial modulins, such as lipopolysaccharide, lipoteichoic acid or murein components (You *et al*., 2008). The classic *Mycoplasma genitalium* membrane consists of two bilipid sheets and membrane proteins instead on these modulins (You *et al*., 2008). The membrane protein has two variants and together it is called lipidassociated membrane proteins (LAMPs) (You *et al*., 2008).

Lipidassociated membrane proteins (LAMPs) are a mixture of mycoplasmal lipoproteins expressed on the surface and it is the main structure for interaction with the host cells (Choi *et al*., 2012; Adamu *et al*., 2013). It is highly antigenic and is the potent targets in inflammatory response (He, *et al*., 2014). *Mycoplasma genitalium* derived LAMPs participate in inflammatory responses and the activation of nuclear factor- κB (NF-κB) in human cell (He, *et al*., 2014). Furthermore, LAMPs can induce the production of pro-inflammatory cytokines, including tumour necrosis factor-a (TNF-a), IL-1b and IL-6 (You *et al*., 2008; He, *et al*., 2014). This overaggressive and inappropriate immune response seems to contribute to the pathogenesis in the *Mycoplasma* infections (You *et al*., 2008).

2.6.6 Clinical manifestation of *M. genitalium* infections

Mycoplasma genitalium infection manifests clinically as urethritis with dysuria or vague urethral discomfort in men (Manhart *et al*., 2008) and to a lesser extent, cervicitis and pelvic inflammatory disease (PID) in women (Korte *et al*., 2006). The incubation period and time to onset of symptoms are unclear (Weinstein and Stiles, 2011). Infection with *M. genitalium* has been strongly linked with acute NGU in men (Jensen, 2004). This observation is particularly strong in cases of NCNGU (Jensen, 2004; Taylor-Robinson and Jensen, 2011). *Mycoplasma genitalium* is detected more frequently in men with persistent or recurrent urethritis following

empiric therapy with doxycycline instead of azithromycin (Wikström and Jensen, 2006). *Mycoplasma genitalium* may also be associated with urethritis in women, although data are too limited to draw clear conclusions (Manhart *et al*., 2008; Taylor-Robinson and Jensen, 2011)

Mycoplasma genitalium is a newly recognised STI in women (Mobley *et al*., 2012). Data showing *M. genitalium* as a cause of genital tract diseases in women are emerging (Svenstrup *et al*., 2008; Mobley *et al*., 2012). However, *M. genitalium* has been implicated as an aetiological pathogen of upper and lower genital tract infections and adverse pregnancy outcome (Bjartling *et al*., 2010). *Mycoplasma genitalium* is an independent risk factor for tubal factor infertility and spontaneous preterm labour (Björnelius, 2010). *Mycoplasma genitalium* is motile and hence can ascend from the lower to upper genital tract after sexual transmission (Ross and Jensen, 2006). Several studies have shown the association between detection of the organism and clinical signs and symptoms of PID (Haggerty *et al*., 2006; Bjartling *et al*., 2010; Oakeshott *et al*., 2010). It has been shown that *M. genitalium* can cause salpingitis and cervicitis (Cohen *et al*., 2005; Mobley *et al*., 2012).

2.6.7 Diagnosis of *M. genitalium*

In sexual health clinics, the microbiological diagnosis of *M. genitalium* is rarely made because of the lack of useful, commercially available diagnostic tests (Mobley *et al*., 2012). *Mycoplasma genitalium* can be cultured in 4-sucrose phosphate (4-SP) medium using urethral discharge or cervical specimens (Jensen, 2006; Björnelius, 2010). This approach is extremely difficult, because of the size, slow growth and growth requirements of *M. genitalium* (Tosh *et al.,* 2007). Growth on 4-SP medium can take more than fifty days before colour change can be observed (Jensen, 2006). The serological methods for the detection of *M. genitalium* include; ELISA and immunoblotting (Svenstrup *et al*., 2006; Iverson-Cabral *et al*., 2011). However, these methods are not reliable as it produces false positives due to the cross-reactions between *M. genitalium* and *M. pneumonia*; hence it is not routinely used (Svenstrup *et al*., 2006; Edberg *et al*., 2008).

In many laboratories the detection of *M. genitalium* is dependent on the development and application of NAATs-based assays (PCR and TMA) (Huppert *et al*., 2008; Short *et al*., 2009). Gene targets commonly used for *M. genitalium* PCR assays include the 78 bp region of the *M. genitalium* adhesion protein and a 517 bp region of the 16S rRNA gene (Jensen,

2006; Twin *et al*., 2011). Assays targeting any of these genes are able to detect as few as 25 copies of DNA per reaction *in vitro* (Twin *et al*., 2011). In spite of the wide spread use of NAATs for detecting *M. genitalium*, there is still no accepted 'gold standard' method of *M. genitalium* detection (Weinstein and Stiles, 2011). Hence it is difficult to compare different assays from various international laboratories (Weinstein and Stiles, 2011).

The optimal specimen type used for *M. genitalium* PCR has not been thoroughly assessed and differential detection may show varying bacterial loads between samples (Wroblewski *et al*., 2006). First void urine, as well as vaginal, urethral, cervical and rectal swabs can be used for *M. genitalium* PCR detection (Weinstein and Stiles, 2011). Self-collected specimens are increasingly been used in *M. genitalium* PCR as their sensitivity in detection assays is closely comparable to clinician-collected specimens (Alexander *et al*., 2008; Van der Helm *et al*., 2009; Falk *et al*., 2010).

2.6.8 Treatment of *M. genitalium*

Antibiotics that can reach high concentrations inside cells are potentially active against mycoplasmas (Bébéar, 2012). The following antibiotics have been used for the treatment of *M. genitalium* with varying efficacy because of this property; lincosamides, streptogramins, ketolides (MLSK), tetracyclines and fluoroquinolones (Bébéar, 2012). Tetracyclines are the first-line treatment against urogenital mycoplasma infections (Bébéar, 2012). These classes of antibiotics are also active against bacterial STDs potentially associated with *M. genitalium* (Bébéar *et al.,* 2008; Cazanave *et al.,* 2012).

Moxifloxacin (400 milligrams for 7 or 10 days) has been used as second-line treatment where *M. genitalium* treatment failure has been observed clinically (Yasuda *et al*., 2005; Manhart *et al*., 2011). Moxifloxacin have a better rate of eradication against low concentration of *M. genitalium* (Cazanave *et al.,* 2012). Nevertheless, it is often used as second or third line because of the high cost of the drug (Cazanave *et al.,* 2012).

2.7 Summary

Neisseria gonorrhoeae is a Gram-negative human pathogen and the causative agent of the gonorrhoea (Hamilton and Dillard, 2006; Phillips *et al*., 2012). It is an infection of global importance yet an effective vaccine is not available (Phillips *et al*., 2012). Clinically, *N. gonorrhoeae* infections may present with a wide range of symptoms and can affect anorectal,

conjunctival, pharyngeal and urogenital areas (Miller, 2006). The culture method is still the "gold standard" method for test evaluation and routine laboratory screening of *N. gonorrhoeae* in spite of the availability of more sensitive NAAT methods (Hopkins *et al*., 2010). Currently, the ESCs (cefixime and ceftriaxone) are the only antibiotics recommended as first-line treatment of gonococcal infections because of the rapid development of resistance to penicillins, tetracycline and quinolones (Walker and Sweet, 2011).

Trichomonas vaginalis is the causative agent of trichomoniasis (Benchimol, 2004). Trichomoniasis may be classified as acute, chronic or asymptomatic depending on the severity of the infection (Pattullo *et al*., 2009). The broth culture method is the most common method used for the diagnosis of trichomoniasis (Crucitti *et al*., 2003; Soper, 2004). An incubation period of two to seven days is required to identify *T. vaginalis* with culture. This prolonged period before a diagnosis is made is a major disadvantage of this method (Hobbs and Seña, 2013). Metronidazole is used for the treatment of *T. vaginalis* infections (Wright *et al*., 2010).

Chlamydia trachomatis is a Gram-negative obligate intracellular bacterium (Somani *et al*., 2000) with a unique two phase life cycle, that alternate between EB and the RB (Hogan *et al.,* 2004). Asymptomatic carriage of *C. trachomatis* infection is high in both men and women and thus remains untreated (Van de Laar and Morre, 2007; Brunhama and Rappuolib, 2013). In the laboratory, chlamydia infections can be detected by cell culture, DFA, EIA and NAAT using urine or vaginal swab specimens (Chernesky, 2005). *Chlamydia* infection can be treated with macrolides, quinolone and tetracycline (Manavi, 2006).

Mycoplasma genitalium is a surface parasite that primarily infects the mucous membrane cells of the urogenital track (Jensen, 2006). Infection presents clinically as urethritis with dysuria in men and cervicitis and PID in women (Korte *et al*., 2006; Manhart *et al*., 2008). *Mycoplasma genitalium* is difficult to culture as it is susceptible to desiccation, osmotic change and temperature (Hayflicks, 2010). The clinical laboratory detection of *M. genitalium* is dependent on NAATs-based assays using urine or vaginal swab specimens (Huppert *et al*., 2008; Short *et al*., 2009). Tetracycline is the treatment of choice for *M. genitalium* infection and moxifloxacin recommended in cases where treatment failure is observed (Manhart *et al*., 2011; Bébéar, 2012; Cazanave *et al.,* 2012)

The purpose of this study was to determine the aetiological pathogens of MUS and VDS and the prevalence in the Tshwane region using a real-time multiplex PCR assay. Furthermore, the antimicrobial resistance of *N. gonorrhoeae* clinical isolates to eight antibiotics (penicillin, cefoxitin, ceftriaxone, cefpodoxime, tetracycline, ciprofloxacin, ofloxacin and spectinomycin) and the MICs were determined by using the Kirby Bauer disc diffusion and the E-test methods respectively. Finally, the NG-MAST scheme was used to determine the genetic epidemiology of *N. gonorrhoeae* clinical isolates.

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CHAPTER 3

ANTIMICROBIAL SUSCEPTIBILITY PATTERN AND MOLECULAR EPIDEMIOLOGY OF *NEISSERIA GONORRHOEAE* CLINICAL ISOLATES

The editorial style of the journal: Journal of Sexually Transmitted Infections was followed in this chapter

3.1 Abstract

Gonorrhoea is a major cause of reproductive tract morbidity in Africa. The phenotype and genotype of the causative agent, *Neisseria gonorrhoeae* is constantly changing as a result of its ability to adapt to antibiotic pressure. These changes can be used to characterise gonococcal strains circulating in a given community, thereby identifying patterns of specific strain transmission and guiding control strategies. Periodic monitoring of the prevalence of *N. gonorrhoeae*, its antimicrobial susceptibility pattern (AMS) and molecular epidemiology can provide essential information for updating local syndromic management guidelines and can aid in the early detection of the emergence of drug resistant strains. The aim of this study was to determine the AMS pattern and the molecular epidemiology of *N. gonorrhoeae* clinical isolates in the Tshwane region, Gauteng, South Africa.

Methods: The Kirby Bauer disc diffusion and the Epsilon-test methods (E-test) were used to determine the AMS pattern and minimum inhibitory concentration (MIC) of *N. gonorrhoeae* to 11 antibiotics. The molecular epidemiology of *N. gonorrhoeae* clinical isolates was determined using *N. gonorrhoeae* multi-antigen sequence typing (NG-MAST). An in-house real-time PCR assay was performed to confirm culture-positive *N. gonorrhoeae* isolates.

Results: Resistance to fluoroquinolones increased from 7% in 2004 up to 70% in 2013 and strongly correlated with multidrug-resistant (MDR) isolates. All isolates were susceptible to third-generation cephalosporins, although two isolates showed increased MICs for cefpodoxime and cefotaxime. The NG-MAST revealed novel as well as previously described sequence types (ST). The NG-MAST STs 2652, 4822, 5268 and 8523 have been previously described, while NG-MAST ST 11715, 11716, 11717 and 11718 are novel STs. One of the five novel STs showed a MDR phenotype. The NG-MAST STs 2652 and 4822 have

previously been described in Johannesburg, Gauteng province and ST 4822 was associated with cefixime treatment failure.

Conclusion: This study supports cefixime as the current first-line treatment for *N. gonorrhoeae* in the Tshwane region, Gauteng, South Africa. The molecular epidemiological data obtained in this study underscore the urgency to introduce a surveillance system for monitoring the AMS of *N. gonorrhoeae* in view of the emergence of MDR *N. gonorrhoeae* in the region.

Keywords: *Neisseria gonorrhoeae,* Antimicrobial susceptibility, Cephalosporins, NG-MAST, Multidrug-resistant

3.2 Introduction

Gonorrhoea is the second most common bacterial sexually transmitted infection worldwide with 106.1 million cases among adults estimated across the globe (Unemo *et al*., 2011; Sethi *et al*., 2013). *Neisseria gonorrhoeae* has developed resistance to all antimicrobial agents previously used as first-line treatments; penicillins, tetracycline and fluoroquinolones (Umemo *et al*., 2011). This emerging antimicrobial resistance in *N. gonorrhoeae* is a major obstacle in the control of gonorrhoea (Florence *et al*., 2012). Antibiotic treatment should therefore be confirmed by local, national and international surveillance programmes (Savicheva *et al*., 2007). As a result, there is a continuous need for area-specific research to monitor patterns of antimicrobial resistance (Cao *et al*., 2008).

The sensitivity of *N. gonorrhoeae* to antibacterial agents can be determined by agar dilution, disc diffusion and the E-test methods (Savicheva *et al*., 2007; CLSI, 2014). The agar dilution and E-test method are quantitative test methods that allow measurement of the MIC of an antibiotic (WHO, 2013). The disc diffusion method is a qualitative method for detection of gonococcal resistance to antibiotics (WHO, 2013). The AMS pattern combined with the knowledge of the sequence type (ST) of gonococcal strains circulating in a community and of changes in the prevalent strains can identify patterns of transmission of gonorrhoea and guide treatment and control efforts (Bennett *et al*., 2007; Dangor *et al.* 2010).

The NG-MAST and full- or extended-length *por*B gene sequencing are currently the best methods for fast, highly discriminatory, portable, reproducible and typeable characterisation of all *N. gonorrhoeae* isolates (Sethi *et al*., 2013). These two methods are internationally

recommended (Sethi *et al*., 2013). In the NG-MAST method, fragments from two gonococcal antigen genes under diversifying immune selection: *por* (codes the outer porin) and *tbp*B (codes the β subunit of transferrin-binding protein) are sequenced (Martin *et al*., 2004).

In the Tshwane region, gonococcal epidemiology has been explored in a few studies. These studies used NG-MAST and phenotypic typing methods, such as antimicrobial susceptibility testing, auxotyping, plasmid profiling and serotyping (De Jongh *et al*., 2007; Dangor *et al.*, 2010). Central to the control of this important pathogen is the need for rapid identification, an AMS pattern and a highly discriminatory typing method. The aim of this study was to determine the AMS patterns of the *N. gonorrhoeae* isolates and to use the NG-MAST method to determine the molecular epidemiology of the *N. gonorrhoeae* isolates circulating in the Tshwane region.

3.3 Methods

Self-collected urine and vaginal swab specimens obtained from 200 sexually active men and women attending the antiretroviral and sexually transmitted infection clinics in Tshwane, Gauteng, South Africa with complaints of symptoms suggestive of acute urethritis and vaginal discharge were included in this study. All participants were instructed on how to self-collect urine and vaginal swab specimens following instructions contained in the informed consent forms. Ethical approval was obtained in January 2012 (S2/2012) prior to commencement of this study. This study was conducted at the Department of Medical Microbiology, University of Pretoria, Prinshof Campus, South Africa. Demographic data was not considered in this study.

Gonococcal culture was performed using urine specimens from all male participants (62) and the self-collected vaginal swabs (138). After culturing, the oxidase test (Sigma-Aldrich, USA), Gram staining and examination under the microscope were performed. Standard techniques were used for the culturing of *N. gonorrhoeae* (Martin *et al*., 2007; Mckechnie *et al*., 2009). Briefly, urine sediment and swabs were streaked onto split agar plates, consisting of Thayer-Martin medium on one half and chocolate medium on the other half (Oxoid, West Heidelberg, Australia) and incubated (Heal Force®, Shanghai Lishen Scientific, China) at 35°C in a 5% CO_2 atmosphere. Plates were examined for growth after 24 and 48 hours of incubation. *Neisseria gonorrhoeae* culture positive isolates were confirmed by an in-house real-time PCR assay. Sugar utilisation identification and penicilinase production

confirmation were done with the API NH (bioMérieux, France) test. Penicilinase-producing *N. gonorrhoeae* (PPNG) isolates produce a TEM-1-type β-lactamase, which confers high-level resistance to penicillin G (Lee *et al*., 2011). *Neisseria gonorrhoeae* culture positive isolates were prepared for storage by inoculating a single colony onto a trypticase soy broth (Oxoid, West Heidelberg, Australia) incubation (Heal Force®, Shanghai Lishen Scientific, China) at 35^0C in a 5% CO_2 atmosphere overnight. A 50:50 of broth to 50% (v/v) sterile glycerol (Merk, Germany) was used for long term storage at -70°C.

The oxidase test was performed as follows; a part of a colony was collected with a sterile dry cotton swab and a few drops of oxidase reagent (tetramethyl-p-phenylene diamine hydrochloride) (Sigma-Aldrich, USA) were placed on it. A gonococcal positive colony turned pink and then rapidly deepened to purple indicating the presence of oxidase.

The identity of the *N. gonorrhoeae* isolates was confirmed by the API NH (bioMérieux, France) carbohydrate and enzyme utilisation test. The API NH (bioMérieux, France) procedure was carried out according to the manufacturer's instructions. Briefly, an inoculum of *N. gonorrhoeae* adjusted to 4 McFarland was prepared from a subculture. The strip was prepared, sample identification number written on the lid and it was placed in the incubation box. Each strip was filled with 50 µl of the 4 McFarland inoculum of the isolate to be identified; after 2 to 4 hours of incubation at 37°C (Vacutec, Scientific, South Africa) colour reactions were recorded.

Neisseria gonorrhoeae isolates retrieved from the stock and sub-cultured (SC) on Thayer-Martin agar medium (Oxoid, West Heidelberg, Australia) were used for antimicrobial susceptibility testing. The antimicrobial susceptibility testing of the gonococcal isolates was carried out using the disc diffusion method on a Thayer-Martin agar medium (Oxoid, West Heidelberg, Australia) supplemented with 1% IsoVitalex (Oxoid, West Heidelberg, Australia). The diameter of the inhibition zone was measured in millimetres and interpreted according to the criteria of the Clinical Laboratory Standards Institute (CLSI, 2013). The AMS of *N. gonorrhoeae* clinical isolates to the following antibiotics: penicillin, cefoxitin, ceftriaxone, cefotaxime, cefpodoxime, cefixime, cefepime, tetracycline, ciprofloxacin, ofloxacin and spectinomycin were determined. The MIC of cefixime, cefotaxime, ciprofloxacin, cefpodoxime and penicillin for *N. gonorrhoeae* strains were determined by the E-test (AB Biodisk, Sweden) on a Thayer-Martin agar medium (Oxoid, West Heidelberg, Australia) enriched with 1% IsoVitalex (Oxoid, West Heidelberg, Australia). Table 3.2

shows the MIC breakpoints for the *N. gonorrhoeae* recommended by the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2014).

The disc diffusion and the E-test methods were performed on all culture positive gonococcal isolates and were repeated four times and the averages were used to determine the AMS patterns of the *N. gonorrhoeae* isolates. Two control strains; *N. gonorrhoeae* ATCC 19424 and *N. gonorrhoeae* ATCC 31426 were used to validate the antimicrobial susceptibility testing. The *N. gonorrhoeae* ATCC 19424 isolate was sensitive to: Cefixime 5 µg, ceftriaxone 30 µg, ciprofloxacin 5 µg, penicillin (10 units) and tetracycline 30 µg, while *N. gonorrhoeae* ATCC 31426 is resistant to penicillin (10 units) but sensitive to cefixime 5 µg, ciprofloxacin 5 µg, ceftriaxone 30 µg and tetracycline 30 µg. Multidrug-resistant *N. gonorrhoeae* isolates is defined in this study as; isolates resistant to at least one of the antibiotics belonging to category I (including ceftriaxone, cefixime and spectinomycin) and two or more antibiotics belonging to category II (including penicillin, ciprofloxacin and azithromycin) (Tapsall *et al*., 2009).

The real-time PCR assay was performed as a confirmatory test for the culture positive isolates. The assay was performed on the LightCycler 480 platform (Roche Molecular Diagnostics, Germany) with a final reaction volume of 25 µl. The Qiagen pathogen mix™ with internal control (Qiagen, Germany) master mix was used for the real-time PCR assay. The primers (Table 3.1) targeted the *N. gonorrhoeae por*A pseudogene, which encodes the gonococcal outer membrane protein. The amplification conditions were as follows: *Taq* activation at 95°C for 10 minutes, 40 cycles of denaturation at 95°C for 20 s, annealing/extension at 61.2°C for 60 s and cooling at 40°C for 30 s. An internal, positive and negative control were included in all PCR assays. The pathogen was detected in the following channels on the LightCycler 480 (Roche Molecular Diagnostics, Germany); IC detection in the HEX channel (465 nm to 580 nm) and *N. gonorrhoeae* detection in the FAM channel (440 nm to 510 nm).

A sequence-based method, NG-MAST, was used to determine the diversity of the two outer-membrane proteins; porin protein (*por*B) and transferrin binding protein B (*tbp*B). The NG-MAST PCR assays were performed with the DNA extracted from all *N. gonorrhoeae* culture positive isolates. The DNA extraction was performed using the ZR Fungal/Bacterial DNA MiniPrep® (Zymo Research, USA) following the manufacturer's instructions.

The NG-MAST PCR assays were performed as described by Martin *et al*. (2004). The primers and the amplicon sizes are shown in Table 3.3. The cycling profile for the *por* PCR assay involved initial denaturation at 95ºC for 4 min, followed by 25 cycles of 95ºC for 30 s, 58ºC for 30 s, and 72ºC for 60 s, with a final extension step of 72ºC for 10 min and cooling to 40ºC. The cycling profile for the *tbp*B PCR assay was similar to the *por* PCR assay but with an annealing temperature of 69ºC. Positive and negative controls were included in all the *por* and *tbp*B PCR assays.

After amplification, the PCR products were separated by agarose gel electrophoresis on 2% (m/v) Seakem agarose gels (Lonza, USA) and a 1 kb DNA ladder (ThermoFischer Scientific, USA) was used as size marker. Gel electrophoresis (Elite 300 power pack, Wealtec, South Africa) was done at 85 V for 2 hours in a 1x Tris-Acetate-EDTA buffer (40 mM Tris-HCℓ, 20 mM NaOAc and l mM EDTA, pH 8.5) (Sigma-Aldrich, USA). The products were visualised using a UV trans-illuminator (Ultra Violet Product, USA).

The PCR products that were 737 bp (*por*) and 589 bp (*tbp*B) in size after gel electrophoresis were sent to Inqaba Biotechnology Industries (Pretoria, South Africa) for *por* and *tbp*B sequencing. The sequences were edited with the CLC Main Workbench 6.0 software programme (CLC Bio, Denmark). The edited sequences were uploaded on the NG-MAST website (http://www.ng-mast.net) where the allele numbers of the *por* and *tbp*B genes and sequence types (STs) were assigned using the NG-MAST website.

3.4 Results

A total of 200 samples obtained from 62 male participants and 138 female attending the antiretroviral clinic and the STI clinic in Tshwane, Gauteng, South Africa was included in this study. Culture and microscopy were performed on all 200 specimens (138 vaginal swabs and 62 urine samples) to detect *N. gonorrhoeae*. Gram stained smears showed Gram-negative intracellular diplococci within polymorphonuclear leukocytes (PMNL) in 19% (38/200) of participants.

Fourteen isolates were presumptively positive for *N. gonorrhoeae* using the culture method. The biochemical test and the real-time PCR assay both confirmed the same 10 isolates from the culture-positive isolates to be *N. gonorrhoeae*. The remaining four isolates were *Haemophilus* spp. confirmed by the confirmatory biochemical test (bioMérieux, France).

Six male and four female participants were positive for *N. gonorrhoeae* by these methods. *Neisseria gonorrhoeae* was isolated at equal rates from both clinics 50% (Table 3.4).

The antimicrobial susceptibility testing results for all *N. gonorrhoeae* isolates are shown in Table 3.5 and further illustrated by Figure 3.1. No antibiotic resistance to cefoxitin, cefpodoxime, ceftriaxone, cefixime, cefepime and cefotaxime was observed. Seven isolates were resistant to ciprofloxacin 70% (7/10), eight isolates were resistant to ofloxacin 80% (8/10), six isolates were resistant to both penicillin and spectinomycin 60% (6/10) and all ten isolates were resistant to tetracycline 100% (10/10). All the penicillin-resistant isolates were penicillinase-producing. Multidrug-resistant *N. gonorrhoeae* was observed in 50% (5/10) of isolates. All ten isolates had MIC values (0.015 µg/ml to ≤0.5 µg/ml) within the susceptible range for the recommended first-line treatment (cefixime).

Two isolates 20% (2/10) were susceptible to penicillin while 30% (3/10) of the isolates had a MIC in the range of intermediate (decreased susceptibility) (0.12 µg/ml to 1 µg/ml) for penicillin and 50% (5/10) of isolates were resistant (≥2 µg/ml) to penicillin. Two isolates 20% (2/10) isolates were fully susceptible to ciprofloxacin. The interpretation of the zone of inhibition for the E-test is shown in Table 3.6.

Ten *N. gonorrhoeae* isolates were genotyped using the NG-MAST method and all 10 of these isolates had AMS pattern results. Typing of these isolates revealed eight ST clusters: ST 4822 (two isolates) and ST 11716 (two isolates) while STs 2652, 5268, 8523, 11715, 11717 and 11718 each had one isolate. Sequence types (2652, 4822, 5268 and 8523) have been previously described while the remaining four STs are novel STs (registered at: http://www.ng-mast.net). The *por-tbp*B alleles, the ST and the corresponding AMS pattern of the *N. gonorrhoeae* isolates are displayed in Table 3.7. The gel electrophoresis image of the *por* and the *tbp*B genes of eight isolates are displayed in Figure 3.2.

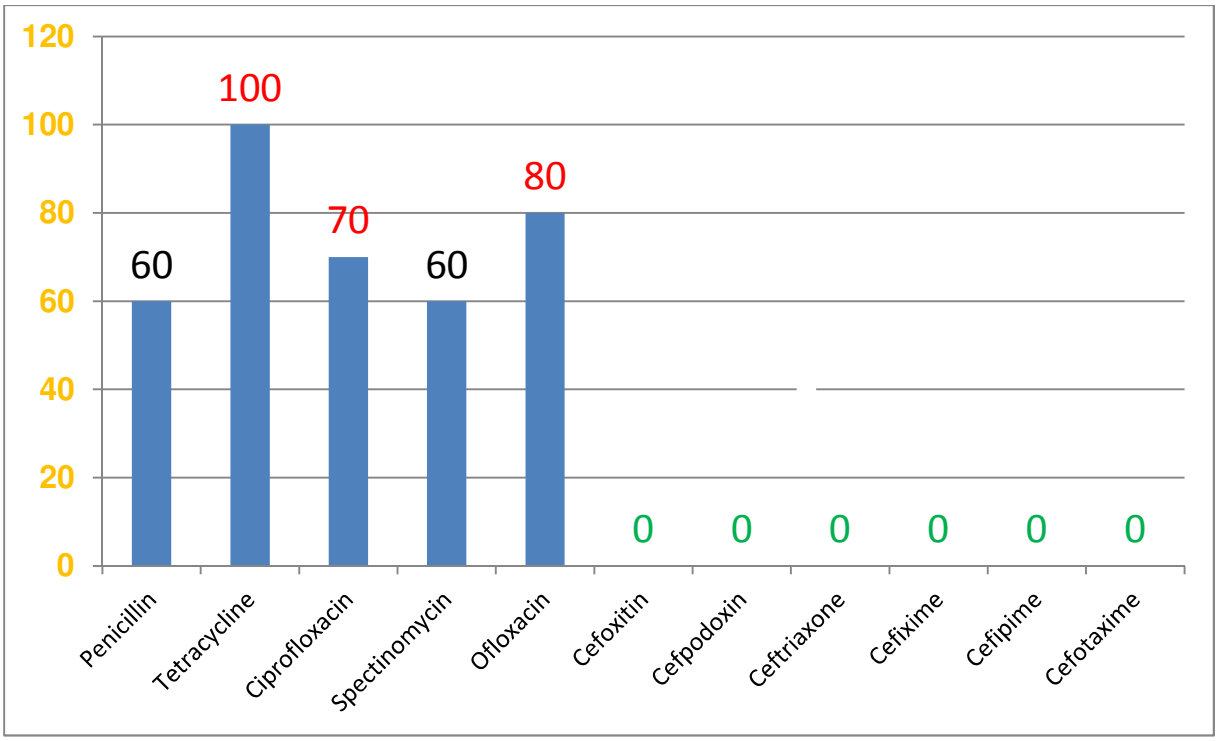

Figure 3.1: The AMS patterns of *N. gonorrhoeae* clinical isolates (n=10) disc diffusion method

3.5 Discussion

Effective treatment of gonococcal infections is essential to prevent adverse outcomes (such as pelvic inflammatory disease (PID) and acute salpingitis) and to interrupt transmission networks (Miller, 2006; Brown *et al*., 2010). Hence, programmes monitoring the antibiotic susceptibility and molecular epidemiology of *N. gonorrhoeae* isolates are essential for the implementation of adequate empirical treatment regimens (Ison and Alexander, 2011; WHO, 2012; Endimiani *et al*., 2014). This study reports the AMS pattern and the molecular epidemiology of *N. gonorrhoeae* isolates obtained from the Tshwane region, South Africa in 2013.

Antimicrobial susceptibility patterns have been previously used to characterise gonococcal isolates in the Tshwane region. In the earlier studies, Dangor *et al.* (2010) reported AMS patterns of gonococcal isolates in the region, over a 20-year period (1984 to 2004), while De Jongh *et al*. (2008) characterised ciprofloxacin resistant isolates in the region using NG-MAST. A comparison of the present study with the earlier studies showed that *N. gonorrhoeae* isolated in clinical settings in the Tshwane region is becoming more resistant and are genetically diverse.

In this study, a high prevalence of 60% (6/10) penicillin resistance was observed. This represented an increase from 25% (35/141) in 2004 to 60% in 2013 in comparison with the Dangor *et al.* (2010) study. Similar high prevalence rates of 62.2% (158/254) and 67.7% (226/334) to penicillin was reported in the studies of Huang *et al*. (2010) and Li *et al*. (2014) conducted in Northern Taiwan and Nanjing, China. A much higher prevalence of 94.4% (27/29) was reported in Ethiopia in the study of Tibebu *et al*. (2013). An increasing trend was observed with the MIC of penicillin in the current study. Contrary to Dangor *et al.* (2010) who reported that 73% of the 141 isolates studied in 2004 had decreased susceptibility (MIC 0.125 to 1 mg/l) to penicillin, 50% (5/10) of the isolates in this study had a MIC well above 2 µg/ml.

All penicillin-resistant isolates in this study were PPNG; this represented an increase from 16% (22/141) in 2004 to 60% in 2013 when compared to the Dangor *et al.* (2010) study. The observation of high and increasing rates of PPNG in this study is similar to the study of Ahmed *et al*. (2010) who reported an increase in the prevalence of PPNG from 28% to 88% during 1997 to 2006 in Bangladesh from 1 767 clinical *N. gonorrhoeae* isolates. The study of Lahra *et al*. (2013) conducted in the Philippines, also reported a high rate [85% (29/34)] of PPNG. In contrast, the European Centre for Disease Prevention and Control (ECDC) reported in 2013 that the rate of high-level PPNG is declining across Europe (ECDC, 2013). The result obtained in the study by Carannante *et al*. (2014) is in support of the ECDC report; the percentage of PPNG significantly declined from 77% in 2003 to 7% in 2012 in Italy. However, in Korea, Lee *et al*. (2011) reported that all 997 isolates were resistant to penicillin despite the decline in PPNG observed in their study. The continued increase in penicillin-resistant *N. gonorrhoeae* isolates despite the decline in PPNG could be as a result of the mutations introduced into the *N. gonorrhoeae* mosaic alleles. This mutation decreases acylation by cephalosporins and also decreases acylation by penicillins (Tomberg *et al*., 2013). Hence, clinical isolates will continue to be resistant to penicillin even though it is no longer used for *N. gonorrhoeae* treatment.

In this study, all isolates were resistant to tetracycline (100%). This represents an increase of 46% in comparison to the Dangor *et al.* (2010) study, in which resistance to tetracycline increased from 7% in 1992 to 54% in 2004. Similar results were obtained in a study conducted in Switzerland by Endimiani *et al*. (2014) where tetracycline-resistant isolates increased from 65.4% (12/26) in 2001 to 88.2% (24/34) in 2012. A similar trend was

observed in the Lee *et al*. (2011) study performed in Korea where tetracycline resistant isolates were between 93% and 100% in 2000 to 2006 from 977 *N. gonorrhoeae* isolates.

There was an increase in the MIC (≥1 µg/ml) of ciprofloxacin in the Tshwane region in 2013 60% (6/10) compared to the De Jongh *et al*. (2007) and the Dangor *et al.* (2010) studies in the same region. Although ciprofloxacin-resistance isolates were reported in both studies the MIC values were very low. The prevalence of quinolone-resistant (QRNG) was 70% (7/10) and 80% (8/10) for ciprofloxacin-resistant and ofloxacin-resistant *N. gonorrhoeae* respectively in this study. This represents a 10 fold increase in less than ten years for ciprofloxacin-resistant *N. gonorrhoeae* when compared with the De Jongh *et al*. (2007) study. This observation is consistent with the findings of Kovari *et al*. (2013) and Endimiani *et al*. (2014) who reported that the prevalence of ciprofloxacin-resistant *N. gonorrhoeae* isolates increased to more than 73% during the last decade in Switzerland. The rate of ciprofloxacin-resistant *N. gonorrhoeae* was also high in Kenya as reported by Duncan *et al*. (2011). These figures showed that the use of this antibiotic for the first-line empirical treatment of gonococcal infections continues to be increasingly ineffective. Ciprofloxacin has been discontinued as the first-line treatment for gonococcal infections in Korea, the Netherlands, South Africa, USA and many other countries, since 2002 (CDC, 2002; Lewis *et al*., 2008; De Vries *et al.,* 2009; Lee *et al*., 2011). However, resistance to quinolones has persisted despite the discontinuation of this antibiotic in many countries.

The prevalence of spectinomycin-resistant isolates was high at 60% (6/10) in this study. This differs from rates reported by Cao *et al*. (2008), who found only seven (5.5%) spectinomycin-resistant isolates in Antananrivo (Madagascar) from 126 gonococcal isolates. Bala *et al*. (2013) reported an increase in spectinomycin resistant isolates from 0.6% (Bhutan) to 10.5% (Kolkata) between 2009 and 2012. In the studies of Dangor *et al.* (2010), Sethi *et al*. (2013) and Carannante *et al*. (2014) all isolates were fully susceptible to spectinomycin in the studies performed in South Africa, Pakistan and Italy respectively.

Spectinomycin or a third generation cephalosporins is recommended for the treatment of uncomplicated *N. gonorrhoeae* with the exception of pharyngeal gonorrhoea (Unemo *et al* 2013). However, spectinomycin is not a promising candidate for first-line empirical treatment of gonorrhoea because of the high cost, limited accessibility and since it easily develops resistance when it is widely used in clinical practice for treatment of gonococcal infections (Lewis, 2011; Ohnishi *et al*., 2011). Therefore, the finding of a high prevalence of

spectinomycin resistant isolates in our setting is surprising, as this antibiotic is not widely used or easily available. Hence, these trends need to be closely monitored.

All isolates (100%) in this study were susceptible to the third generation cephalosporins tested (cefoxitin, cefpodoxime, ceftriaxone, cefixime, cefipime and cefotaxime), although two (20%) of the isolates had slightly higher MICs for cefpodoxime (0.016 µg/µl and 2 µg/µl) and cefotaxime (0.75 µg/µl and 0.032 µg/µl) compared to the other eight isolates. These results are consistent with regional studies, which show that this class of antibiotic is still effective for the management of gonococcal infections. The studies of Lewis *et al*. (2008), Apalata *et al*. (2009) and Brown *et al*. (2010) performed in South Africa, Mozambique and Malawi reported 0% prevalence for ceftriaxone, ceftriaxone/ cefixime and ceftriaxone respectively. Earlier studies by De Jongh *et al*. (2007) and Black *et al*. (2008) in South Africa reported similar prevalence rates for ceftriaxone/ cefpodoxime and ceftriaxone.

The two isolates reported in this study with increased MICs (decreased susceptibility) for cefpodoxime and cefotaxime were, however, susceptible to cefixime. The finding of decreased susceptibility to third generation cephalosporins is consistent with the results of the Tanaka *et al*. (2011) study performed in Fukuoka, Japan. Tanaka *et al*. (2011) reported a high rate [61.6% (149/242)] of isolates with decreased susceptibility to the oral cephalosporins and 14.0% (34/242) of isolates with decreased susceptibility to the injectable cephalosporins. Similarly, Lewis *et al*. (2012) reported two gonococcal isolates in the Gauteng province, South Africa, which exhibited decreased susceptibility to cefixime (MIC 0.125 mg/l) and cefpodoxime (MIC 0.75 mg/l and MIC 1 mg/l).

Extended-spectrum cephalosporins (ESCs) (ceftriaxone and cefixime) have replaced quinolones as the first-line treatment of gonococcal infections in Canada and the United States of America (Allen *et al*., 2011). In Ghana, Namibia and South Africa cefixime is now used as the first-line presumptive treatment for gonorrhoea (Lewis *et al*., 2011). However, there have been reports of rapidly emerging resistant gonococcal isolates to these antimicrobial agents. Lewis *et al*. (2013) reported the first two gonococcal isolates resistant to cefixime in the Gauteng province, South Africa. Chen *et al*. (2014) reported that 5.4% (11/198) of isolates in their study conducted in China were resistant to ceftriaxone. *Neisseria gonorrhoeae* isolates with high-level resistance to ceftriaxone and cefixime have been described in the studies of Ohnishi *et al*. (2011), Camara *et al*. (2012) and Unemo *et al*. (2012) in Japan, Catalonia (Spain) and Sweden respectively. As reports of *N. gonorrhoeae*

isolates resistant to/or with decreased susceptibility to the cephalosporins gradually accumulate around the world, could this be a sign that the age of untreatable gonococcal infection is close (Unemo *et al*., 2012).

Fifty percent (5/10) of the *N. gonorrhoeae* isolates in this study had a MDR phenotype. This finding is similar to that of Ahmed *et al*. (2010) who reported that 44% of their studied isolates were MDR *N. gonorrhoeae*. Allen *et al*. (2011) reported a much lower prevalence of 18% (28/149) of MDR *N. gonorrhoeae* in their study in Ontario, Canada.

The NG-MAST STs 11717 and 11718 are both sensitive to penicillin (10 units) and has decreased susceptibility to ciprofloxacin (5 µg). Sequence type 11715 has decreased susceptibility to penicillin (10 units) but is resistant to ciprofloxacin (5 µg) while both isolates belonging to ST 11716 had a MDR phenotype. The NG-MASTs ST 2652 and ST 4822 reported in this study both have been previously described in Johannesburg, Gauteng province, South Africa. Similar to the Magooa (2010) study, ST 2652 continues to be sensitive to ciprofloxacin five years after it was first described in Johannesburg. The two isolates with NG-MAST ST 4822 both have a MDR phenotype with a MIC of 3 µg/ml for ciprofloxacin and were both susceptible to ceftriaxone and cefixime. One of the isolates belonging to NG-MAST ST 4822 was isolated from a female participant and had a high MIC for cefpodoxime (2 µg/µl). This finding of high cefpodoxime MIC and MDR phenotype is consistent with the ST 4822 that was first reported in the study of Lewis *et al*. (2012). Contrary to Lewis *et al*. (2013) study, the ST 4822 strains reported in the current study were isolated from a female and a male participant whose sexual orientation is unknown, while the ST 4822 stains described in the Lewis *et al*. (2013) study were isolated from two homosexual men.

The two *N. gonorrhoeae* isolated from the homosexual men in the Lewis *et al*. (2013) study were identical, differing only by a single nucleotide polymorphism (SNP) in the full-length *por*B gene and has multilocus sequence typing (MLST) sequence type ST 1901. Furthermore, the isolates had the type XXXIV *pen*A mosaic allele and resistance mutations in the *mtr*R (multiple transferrable resistance regulator) promoter (A-deletion) (Lewis *et al*. 2013). The presence of a *pen*A mosaic allele with mutations at position A501 have been associated with increased ESC MICs (Whiley *et al*., 2007; Lewis, 2010; Unemo and Nicolas, 2012; Unemo *et al*., 2012). The PCR determination of the mosaic sequence in the *pen*A gene that encodes for a mosaic penicillin-binding protein 2 (PBP 2) and the PCR-based

amplification and sequencing of additional resistance determinants, such as the *mtr*R (including its promoter), was not performed in this study.

The NG-MAST ST1407, an international clone with decreased susceptibility/resistance to ESC has been identified in Australia, Japan, Norway, Sweden and many other countries (Golparian *et al*., 2010; Unemo *et al*., 2010; Tanaka *et al* 2011; Unemo *et al*., 2011; Unemo and Nicolas, 2012; Nguyen *et al*., 2014) was not identified in this study. Based on the *tbp*B and *por* allele numbers, two isolates (M12 and MS24) belonged to an identical NG-MAST ST 11716. These two isolates belong to the same genogroup; genogroups are defined as having identical *tbp*B alleles and ≥99% DNA sequence similarity within *por*B alleles as defined by NG-MAST (Nguyen *et al*., 2014). This indicates that a genogroup circulating between the two populations studied has been identified in this study. Furthermore, this genogroup has similar AMS patterns regardless of the population.

This study has several noteworthy limitations: The sample size is small and does not represent all cases of *N. gonorrhoeae* in the two clinics. Secondly, genotyping was performed on a limited number of isolates; hence concrete epidemiological conclusions cannot be drawn. The other limitation of this study is the absence of demographic data, such as sexual orientation and individually linked clinical cure data.

3.6 Conclusion

The results of this study support the current first-line treatment for *N. gonorrhoeae* (cefixime) in South Africa. Associations between *N. gonorrhoeae* AMS patterns and NG-MAST STs (*por-tbp*B allele numbers) were identified and may be useful in predicting AMS patterns in future. Despite the small population of *N. gonorrhoeae* studied, NG-MAST analysis showed a diversified population of *N. gonorrhoeae* isolates in the Tshwane region, South Africa. The presence of the MDR strain, ST4822, in Tshwane provided evidence of its provincial distribution. The high number [80% (8/10)] of novel STs identified in this study speaks directly to the limited number of NG-MAST studies done in this region. The need for a surveillance system for continuous monitoring of the AMS patterns of *N. gonorrhoeae* in view of the emergence of MDR *N. gonorrhoeae* in the region is strongly advocated.

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Table 3.1: Nucleotide sequences of the primers and probes used in the real-time PCR assay for the detection of *N. gonorrhoeae* (Goire *et al*., 2008)

Specific target	Primer-Probe Sequence (5'→3')
papTM-F	CAG CAT TCA ATT TGT TCC GAG TC
papTM-R	GAA CTGG TTT CAT CTG ATT ACT TTC CA
papTM-P	HEX-CGCCTATACGCCTGCTACTTTCACGC- Zen Iowa Black

Table 3.2: MIC breakpoints for antimicrobial susceptibility of *N. gonorrhoeae* isolates recommended by the CLSI (Deguchi *et al.,* 2010; CLSI, 2014)

Antimicrobial Agent	Breakpoint MIC (µg/ml)		
	Susceptible	Intermediate	Resistant
Penicillin G	0.06 or less	0.12–1	2 or greater
Cefixime	0.25 or less	—	—
Ceftriaxone	0.25 or less	—	—
Ciprofloxacin	0.06 or less	0.12–0.5	1 or greater

Table 3.3: Nucleotide sequences of the primers used for the PCR assay and sequencing of the *por* and the *tbp*B genes (Martin *et al*., 2007)

Primer name	Sequence (5'-3')	Amplicon size (bp)
Por-Mast1-F	CAA GAA GAC CTC GGC AA	737
Por-Mast2-R	CCG ACA ACC ACT TGG T	
*tbp*BMast1-F	CGT TGT CGG CAG CGC GAA AAC	589
*tbp*BMast2-R	TTC ATC GGT GCG CTC GCC TTG	

Table 3.4: Age and clinic distribution of all *N. gonorrhoeae* culture positive isolates

Sample ID	M11	M12	MS31	F25	F26	MS007	MU007	MS24	VS025	VS048
HIV clinic STD clinic	*	*	*	*	*	*	*	*	*	*
Age	30	41	33	46	27	35	35	60	20	25

Table 3.5: Interpretation of zones of inhibition for the disc diffusion experiments

Antibiotics	PC19424	PC31426	M12	F25	F26	VS025	VS048	MS24	M11	MS007	MU007	MS31
Penicillin 10 units	S	R	R	R	I	R	R	R	R	I	S	S
Cefoxitin 30 µg	S	S	S	S	S	S	S	S	I	S	S	S
Cefpodoxime 10 µg	S	S	S	S	S	S	S	S	S	S	S	S
Ceftriaxone 30 µg	S	S	S	S	S	S	S	S	S	S	S	S
Tetracycline 30 µg	S	S	R	R	R	R	R	R	R	R	R	R
Ciprofloxacin 5 µg	S	S	R	R	R	R	R	R	S	R	I	I
Spectinomycin 25 µg	R	I	R	R	S	R	R	R	S	R	S	S
Ofloxacin 5 µg	S	S	S	S	R	R	R	S	I	R	R	R
Cefixime 5 µg	S	S	S	S	S	S	S	S	S	S	S	S
Cefipime 30 µg	S	S	S	S	S	S	S	S	S	S	S	S
Cefotaxime 30 µg	S	S	S	S	S	S	S	S	S	S	S	S

S: sensitive, I: intermediate, R: resistant

Table 3.6: Interpretation of zones of inhibition of the E-test experiments

Antibiotics	PC19424	PC31426	M12	F25	F26	VS025	VS048	MS24	M11	MS007	MU007	MS31
Penicillin 0.016-259 µg/ml	S 0.023	R 12	R 3	R 3	I 0.125	R 16	I 1	R 24	R 5	I 1	S 0.0125	S 0.032
Cefixime 0.016-256 µg/ml	S 0.016	S 0.016	S 0.023	S 0.016	S 0.016	S 0.016	S 0.047	S 0.016	S 0.016	S 0.047	S 0.047	S 0
Cefpodoxime 0.016-256 µg/ml	S 0.016	S 0.023	S 0.016	S 0.016	S 0.016	S 0.047	S 2	S 0.016	S 0.016	S 0.016	S 0.016	S 0.016
Cefotaxime 0.032-256 µg/ml	S 0.016	S 0.016	S 0.016	S 0.75	S 0.016	S 0.023	S 0.032	S 0.016	S 0.016	S 0.125	S 0.125	S 0.016
Ciprofloxacin 0.002-32 µg/ml	S 0.03	S 0.004	I 0.50	R 2	R 3	R 1	S 0	S 0.50	S 4	S 0	S 0.004	I 0.19

S: sensitive, I: intermediate, R: resistant

Table 3.7: Genetic characteristics and the corresponding AMS patterns of *N. gonorrhoeae* isolates in Tshwane in 2013

Isolates	*Por* allele	*tbp*B allele	ST	PEN	TET	CIP	CFM	CRO	SPC
F25	19	25	5268	R	R	R	S	S	R
F26	6878 (N)	623	11715 (N)	I	R	R	S	S	S
M11	121	165	2652	R	R	S	S	S	S
M12	6879 (N)	137	11716 (N)	R	R	R	S	S	R
MS007	1903	29	4822	I	R	R	S	S	R
MS24	6879 (N)	137	11716 (N)	R	R	R	S	S	R
MS31	6880 (N)	15	11717 (N)	S	R	I	S	S	S
MU007	35	2005 (N)	11718 (N)	S	R	I	S	S	S
VS025	5071	137	8523	R	R	R	S	S	R
VS048	1903	29	4822	R	R	R	S	S	R

PEN: Penicillin, TET: Tetracycline; CIP: Ciprofloxacin; CFM: Cefixime; CRO: Ceftriaxone; SPC: Spectinomycin; N: Novel

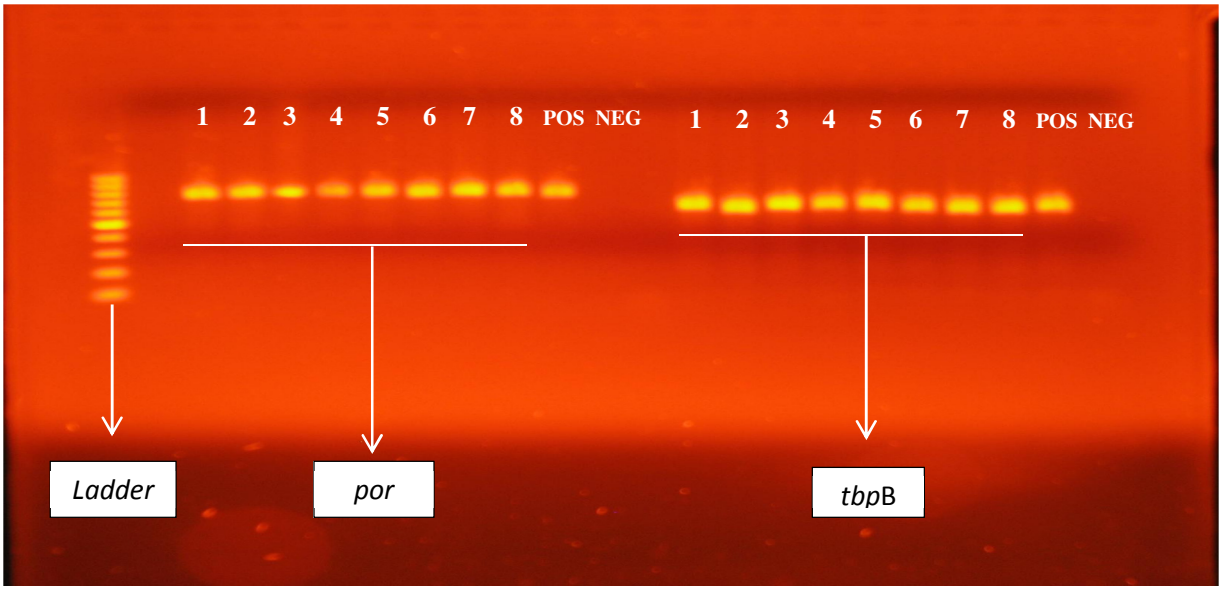

Figure 3.2: **Gel electrophoresis image of the *por* (737 bp) and *tbp*B (589 bp) genes of eight isolates. Bands for specimens: M12, F25, F26, VS025, MS25, MS007, MU007 and MS31 are shown in lanes numbered 1 to 8.**

CHAPTER 4

MULTIPLEX REAL-TIME PCR ASSAY FOR THE DETECTION OF *CHLAMYDIA TRACHOMATIS*, *MYCOPLASMA GENITALIUM, NEISSERIA GONORRHOEAE* AND *TRICHOMONAS VAGINALIS* FROM SELF-COLLECTED SPECIMENS

The editorial style of the journal: BMC Infectious Diseases was followed in this chapter

4.1 Abstract

Sexually transmitted infections (STIs) are a major cause of morbidity in sexually active people and continue to present a leading medical, social and economic problem worldwide. Globally, an estimated 340 million cases of curable STIs, which include chlamydial infection, gonorrhoea, trichomoniasis and syphilis occur annually with an incidence that is increasing in many parts of the world. Both men and women can be affected by STIs. Most STIs are asymptomatic but the consequences are serious. Undiagnosed and untreated STIs can lead to long-term complications. The prevention and early treatment of STIs are key public health priorities in South Africa. However, this effort is hampered by the lack of rapid diagnostic methods. The gold standard for diagnosing STIs, such as chlamydial, gonococcal and trichomoniasis infections remains the culture method. However, the causative pathogens of these STIs are fastidious and do not survive very long outside the host. Furthermore, culturing requires specimens that is often invasive and will take days to result. The aim of this study was to use a real-time multiplex polymerase chain reaction (PCR) assay to simultaneously detect *Chlamydia trachomatis, Mycoplasma genitalium, Neisseria gonorrhoeae* and *Trichomonas vaginalis*, the aetiological pathogens of male urethritis syndrome (MUS) and vaginal discharge syndrome (VDS), using self-collected urine and vaginal swab specimens obtained from male and female participants respectively.

Methods: Two hundred male and female (62:138) adults attending the antiretroviral and sexually transmitted infections clinics in Tshwane, Gauteng, South Africa complaining of symptoms suggestive of vaginal discharge and acute urethritis were included in this study. Vaginal discharge and first-void urine samples were subjected to wet-mount microscopy, Gram stained smears and gonococcal culture for the detection of *N. gonorrhoeae* and *T. vaginalis* respectively. A real-time multiplex PCR assay was used for the molecular detection of *C. trachomatis, N. gonorrhoeae, M. genitalium* and *T. vaginalis*.

Results: The prevalence of MUS and VDS pathogens in this study was 57% (113/200). Pathogens responsible for urethritis were diagnosed in 77% (48/62) of male participants while VDS were found in 47% (65/138) of the female participants. In comparison to the gonococcal culture method the real-time multiplex PCR assay showed sensitivity of 100% and specificity of 75% for *N. gonorrhoeae* for both urine and vaginal swabs.

Conclusion: The findings of this study showed that *N. gonorrhoeae* is the most common cause of urethritis and vaginal discharge in the Tshwane region followed by *T. vaginalis*. The real-time multiplex PCR assay was rapid and suitable for the detection of all four pathogens at relatively low cost due to multiplexing. The results also demonstrated that it is necessary to test for all four pathogens namely *C. trachomatis*, *M. genitalium*, *N. gonorrhoeae* and *T. vaginalis* in patients presenting with urethritis or vaginal discharge in the Tshwane region.

Key words: *Chlamydia trachomatis,* Multiplex real-time PCR assay, *Mycoplama genitalium, Neisseria gonorrhoeae*, self-collected urine, self-collected vaginal swab, *Trichomonas vaginalis*

4.2 Background

Genitourinary infections, including STIs, are caused by a large number of different microbial agents that cause considerable morbidity and mortality worldwide (Amin *et al*., 2007). Urethritis and vaginal discharge syndrome are the most common STI syndromes diagnosed in men and women (Amin *et al*., 2007; Mhlongo *et al*., 2010; Sonkar *et al*., 2014). Urethritis occurs in both men and women but often is unrecognised in women (Mckechnie *et al*., 2009). It is broadly categorised as non-gonococcal urethritis (NGU) or gonococcal urethritis and it is characterised by discharge and dysuria (Mckechnie *et al*., 2009: Tsai and Li, 2013). The pathogens implicated in these two syndromes include *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and *Trichomonas vaginalis*, whilst the role of *Mycoplasma genitalium* and *Ureaplasma urealyticum* in these syndromes remains minimal (Mhlongo *et al*., 2010).

The South African sexually transmitted diseases (STD) syndromic management algorithm recommends the presumptive management of these syndromes with cefixime or ceftriaxone with doxycycline and metronidazole (Lewis and Maruma, 2009; Le Roux *et al*., 2010). This approach is cheap, allowing nurses to treat these STIs without the need for microbiological diagnosis (Mhlongo *et al*., 2010). In spite of these advantages, this approach can still result in a very high rate (98%) of overtreatment for chlamydial and gonorrhoeae infections in women presenting with vaginal discharge syndrome (Peeling, 2009). Hence, efforts should be made in improving diagnostic methods in order to obtain reliable data on the aetiology of major STI syndromes (Morency *et al*., 2001). This effort will ensure that the flow chart performs better and to develop interventions for patients whose symptoms have not improved despite the proper use of recommended drugs (Morency *et al*., 2001).

The World Health Organization (WHO)'s STI diagnostics initiative supports the development of cheap, reliable, non-invasive and easy-to-use tests, especially for the diagnosis of chlamydia and gonorrhoea (Gaydos *et al*., 2006). There are a number of methods available for detecting *C. trachomatis*, *M. genitalium, N. gonorrhoeae* and *T. vaginalis*, which includes: Bacterial culture, enzyme-linked immunosorbent assays (ELISA) for antigen or antibody detection (Clad *et al*., 2000) and molecular diagnostic assays (Chan *et al*., 2000; Chernesky *et al*., 2005; Muvunyia *et al*., 2011).

Molecular assays, such as PCR assays, transcription mediated amplification (TMA), etc. have been used to detect sexually transmitted pathogens in diagnostic laboratories, but PCR assays have been found to be highly sensitive for detecting these pathogens (Le Roux *et al*., 2010;

Muvunyia *et al*., 2011; Ginocchio *et al*., 2012). The high level of sensitivity of PCR assays, allows the use of less invasive specimen types, including first-void urine specimens or self-collected vaginal swabs that are usually unsuitable for less sensitive methods, such as culture and antigen tests (Mckechnie *et al*., 2009).

A multiplex PCR assay has an advantage over single PCR assays in screening, since it involves the simultaneous detection of multiple pathogens (Muvunyia *et al*., 2011). Furthermore, the inclusion of an internal control reaction in a multiplex assay identifies the possible presence of PCR inhibition (Muvunyia *et al*., 2011).

Previously, multiplex PCR-based assays have been used to determine the prevalence of different combinations of the pathogens responsible for urethritis and vaginal discharge syndrome. These assays include; multiplex PCR-based reverse line blot (RLB) assay (Muvunyia *et al*., 2011), "STDFinder" assay, a novel multiplex ligation-dependent probe amplification (MLPA) assay (Muvunyia *et al*., 2011) and a real-time multiplex PCR assay, developed at the Centers for Disease Control and Prevention (CDC) (Mhlongo *et al*., 2010). Commercially available assays are often used for the simultaneous detection of *C. trachomatis* and *N. gonorrhoeae* and this include Abbott *m*2000 real-time™ CT/NG assay, Cepheid CT/NG Xpert rapid PCR, BD Probe Tec and Roche Cobas® 4800 (Le Roux *et al*., 2010; Gaydos *et al*., 2013; Geelen *et al*., 2013; Gueye *et al*., 2014; Han *et al*., 2014). These real-time PCR platforms are often closed systems, expensive and are technically demanding.

In this study a real-time multiplex PCR assay was optimised using previously published primers/probes. The real-time multiplex PCR assay was used to determine the prevalence of *C. trachomatis, M. genitalium, N. gonorrhoea* and *T. vaginalis* using self-collected vaginal and urine specimens obtained from female and male participants respectively, who were attending the antiretroviral and sexually transmitted infections clinics in Tshwane, Gauteng, South Africa.

4.3 Methods

The study was performed at the Department of Medical Microbiology, University of Pretoria from August 2012 to October 2014. Ethical approval was obtained in January 2012 (S2/2012) prior to commencement of the study. This was a prospective study in which the study population included two hundred sexually active men and women (62:138) with signs of urethral and vaginal discharge syndromes attending the antiretroviral and sexually transmitted infections clinics in Tshwane, Gauteng, South Africa. Sample size calculations were performed using an expected prevalence of 50% to an accuracy within 10% with a 95% confidence interval.

Informed consent was obtained from all participants before any specimens were collected. A volume of 25 ml first-void urine specimen were collected from the 62 male participants while two self-collected vaginal swabs (Copan E-swab, Copan Diagnostics, Inc, Italy) were collected from the 138 female participants during a single visit to the clinic.

The E-swab (Copan Diagnostics, Inc, Italy) is a flocked nylon swab, which contain Amies transport medium. Shortly after collection at the site, the swab (Copan Diagnostics, Inc, Italy) was inoculated into the transport medium, stored in a cooler box with ice and transported in batches daily to the research laboratory of the Department of Medical Microbiology, University of Pretoria/NHLS, South Africa where further processing was conducted. Participants with diagnosed STI syndromes were treated by the attending clinicians at the clinic according to the national guidelines, which recommend the following care; antimicrobial agents, partner notification, condom distribution and voluntary counselling testing (VCT) promotion on the same visit (Black *et al*., 2008).

A portion of the first-void urine and one of the vaginal swabs were used to prepare a wet-preparation for direct wet-mount microscopy of *T. vaginalis* before inoculation onto Thayer-Martin and into modified Diamond's culture media for culturing *N. gonorrhoeae* and *T. vaginalis* respectively. The identity of the *N. gonorrhoeae* isolates was confirmed by the API NH (bioMérieux, France) carbohydrate and enzyme utilisation test. The procedure was carried out according to the manufacturer's instructions.

A volume of 200 µℓ of the urine specimens from the male participant and the second vaginal swab were used for the real-time multiplex PCR assay detection of *C. trachomatis*, *M. genitalium, N. gonorrhoeae* and *T. vaginalis*. The specimens for the real-time multiplex PCR

assay were stored at 2ºC to 8ºC; whereas consumables were stored at room temperature (±25ºC) until DNA extraction was performed. The DNA extraction was performed using the ZR Fungal/Bacterial DNA MiniPrep® (Zymo Research Corporations, Thermo Scientific, USA) following the manufacturer's instructions.

The real-time multiplex PCR assay was performed on the LightCycler 480 platform (Roche Molecular Diagnostics, Germany) in a final reaction volume of 25 µl. The Qiagen pathogen mix™ with internal control (Qiagen, Germany) was used for the real-time multiplex PCR assay. The primers (Table 4.1) targeted the *N. gonorrhoeae por*A pseudogene (encodes the gonococcal outer membrane protein), the cryptic plasmid of *C. trachomatis*, the 16S rRNA gene of *M. genitalium* and the *T. vaginalis* specific 2-Kb repeat DNA sequences. The oligonucleotide primers and the TaqMan probes were synthesised by Inqaba Biotechnical Industries, South Africa.

The amplification conditions were as follows: *Taq* activation 95ºC (10 minutes), 40 cycles of the following PCR steps, which included: denaturation (95ºC, 20 s), annealing / extension (61.2ºC, 60 s) and cooling (40ºC, 30 s). The amplification conditions and the real-time multiplex PCR assay were optimised and validated by using two *N. gonorrhoeae* reference strains (*N. gonorrhoeae* ATCC 19424 and *N. gonorrhoeae* ATCC 31426), *C. trachomatis* elementary bodies obtained from the Institute of Tropical Medicine (Antwerpen, Belgium), AmpliRun *M. genitalium* DNA control (Vircell SL, Spain) and *T. vaginalis* DNA that were positive from a previous study in the Department of Medical Microbiology, University of Pretoria/NHLS, South Africa

A sample was considered positive for gonorrhoea or trichomoniasis if the culture and the confirmatory biochemical result were positive or a positive PCR result or both. Gram-negative diplococci detected on microscopy, in the absence of a positive gonococcal culture and/or real-time multiplex PCR result, were regarded as a false positive finding. Internal, positive and negative controls were included in all real-time multiplex PCR assays.

The pathogens were detected in the following channels on the LightCycler 480 (Roche Molecular Diagnostics, Germany); internal control detection in the HEX channel (465 nm to 580 nm), *N. gonorrhoeae* detection in the FAM channel (440 nm to 510 nm), *C. trachomatis* detection in the CAL 590 channel (498 nm to 610 nm), *T. vaginalis* detection in the Cy5 (618 nm to 660 nm) and *M. genitalium* detection in the CAL 610 channel (533 nm to 640 nm). A positive result was accepted in the real-time multiplex PCR assay when a sample showed

amplification fluorescence and a crossing point of ≤ 35 cycles (calculated by the LightCycler Software version 1.5.0).

4.4 Results

A total of 200 samples were included in this study, which included 50 female and 50 male participants enrolled at the STI clinic and 88 females and 12 male participants enrolled at the antiretroviral clinic. The human immunodeficiency virus (HIV) status of participants from the STI clinic is unknown. *Trichomonas vaginalis* culture was performed on the first 40 specimens obtained from the STI clinic but discontinued in the subsequent specimens as it did not add to the diagnostic yield. Bacterial culture and microscopy were performed on all 200 specimens to detect *N. gonorrhoeae*. The multiplex real-time PCR assay was performed on all participants' samples. Testing and validation with clinical isolates and reference strains of target organisms showed that each target species was amplified in the real-time multiplex PCR assay. The number of specimens that tested positive and negative with culture, microscopy and the real-time multiplex PCR assay for *N. gonorrhoeae* are displayed in Table 4.2.

The wet-mount microscopy examination and culture in Diamond's media revealed 8% positive *T. vaginalis* (3/40) among the studied cases. Gram-stained smears showed Gram-negative intracellular diplococci/cocci in 19% (38/200) of the isolates. Fourteen isolates [7% (14/200)] were positive gonococcal isolates by culture method, but the biochemical test confirmed only 10 of these isolates to be *N. gonorrhoeae*. The remaining four [2% (4/200)] isolates were *Haemophilus* spp. as confirmed by the confirmatory biochemical test (API NH, bioMérieux, France). Hence, 5% (10/200) of the 200 studied samples were positive gonococci confirmed by both culture and the biochemical tests.

The real-time multiplex PCR assay was able to detect at least one pathogen in 57% (113/200) of the participants, while no pathogen was detected in the remaining 43% (87/200) of participants. Overall 29% (57/200) *N. gonorrhoeae,* 11% (22/200) *T. vaginalis*, 10% (19/200) *C. trachomatis* and 8% (15/200) *M. genitalium* were detected in the specimens. In total, 16% (18/113) of the participants harboured more than one pathogen at the same time (mixed infection). The mixed infections were distributed as follows: 39% (7/18) had *N. gonorrhoeae* and *T. vaginalis*, 33% (6/18) of the participants had *C. trachomatis* and

N. gonorrhoeae and 17% (3/18) had *C. trachomatis* and *M. genitalium*, while 11% (2/18) had *C. trachomatis* and *T. vaginalis*.

The prevalence of urethritis in male participants were 48% (30/62) *N. gonorrhoeae*, 9.7% (6/62) *T. vaginalis*, 9.7% (6/62) *C. trachomatis* and 9.7% (6/62) *M. genitalium*. The prevalence of vaginal discharge pathogens were 19.6% (27/138) *N. gonorrhoeae*, 11.6% (16/138) *T. vaginalis*, 9.4% (13/138) *C. trachomatis* and 6.5% (9/138) *M. genitalium*. The prevalence according to the HIV status was as follows: STI clinic participants (unknown HIV status); 49% (49/100) *N. gonorrhoeae*, 15% (15/100) *T. vaginalis*, 8% (8/100) *C. trachomatis* and 0% *M. genitalium*. The prevalence of urethritis and vaginal discharge pathogens in the antiretroviral clinic (HIV positive participants) was as follows: 15% (15/100) *M. genitalium*, 11% (11/100) *C. trachomatis*, 8% (8/100) *N. gonorrhoeae* and 7% (7/100) *T. vaginalis*. The prevalence comparison between the two clinics, gender and the overall infection rate is displayed in Table 4.3, while pathogen detection according to symptoms by the real-time multiplex PCR assay is indicated in Figure 4.1.

4.5 Discussion

Before 2010, no national representative community-based microbiological data for STIs were available, except for the seroprevalence of HIV and syphilis in South Africa (Mhlongo *et al*., 2010). The ultimate aim of the South African National Microbiological Surveillance (NMS) programme for STIs is to inform the national STI programme about the appropriateness of existing STI treatment algorithms (Mhlongo *et al*., 2010).

This study is the first to determine the prevalence of the aetiological pathogens of MUS and VDS in the Pretoria region, Gauteng, South Africa using a real-time multiplex PCR assay. The study population included both female and male HIV positive participants, which provided the opportunity to compare the co-infection of these important pathogens with HIV. Demographic data was not considered in this study, however, the two healthcare facilities included, is frequented by black, white and resident foreign communities alike.

In this study, all participants were identified by the healthcare givers / nurses after physical examination was performed and would have all qualified for treatment under the current STI syndromic management approach. However, according to the real-time multiplex PCR assay this management approach would have resulted in up to 43% overtreatment in the study population. Overtreatment increases the financial burden on the public healthcare system, increasing the chance of microorganisms developing resistance. This study showed that this

management approach is not effective. Peeling (2009) reported that the syndromic STI management approach can result to as much as 98% overtreatment for chlamydia and gonorrhoea in women presenting with vaginal discharge syndrome. These findings is consistent with the study conducted by White *et al.* (2008) in rural KwaZulu-Natal, South Africa, in which syndromic STI treatment services cured only 13% of symptomatic curable STI episodes. The study further attributed the continuing high prevalence of curable STIs in rural KwaZulu-Natal to the low effectiveness of the STI management approach. This is in agreement with Hovhannisyan *et al.* (2013) who reported that algorithms for the management of vaginal discharge syndrome had poor to moderate performance for the treatment of chlamydia and gonococcal infections in symptomatic women.

Abnormal vaginal discharge is classified as either physiological or pathological (Mitchell, 2004); women often self-treat or seek medical help for this syndrome. It is not uncommon for healthcare providers to miss the correct diagnosis of vaginal discharge without the proper laboratory test (Yusuf *et al.,* 2011). The pathogens responsible for abnormal vaginal discharge were found in 47% (65/138) of the cases in this study. *Neisseria gonorrhoeae* [19.6% (27/138)] was the most common microbiological cause in the study population, followed by *T. vaginalis* [11.6% (16/138)], *C. trachomatis* [9.4% (13/138)] and *M. genitalium* [6.5% (9/138)]. Mixed infections were observed in 17% (11/65) of participants and no pathogen was diagnosed in 53% (73/138) of the participants.

The high rate (53%) of undiagnosed vaginal discharge could be as a result of physiological discharge, hence no pathogen was detected. Another possible reason may be due to conditions, such as bacterial vaginosis and vulvovaginal candidiasis, which can cause this condition but which was not investigated in this study. In the study of Yusuf *et al*. (2011) pathogens responsible for VDS were found in 94.8% (237/250) of the sexually active females attending the out-patient department (OPD) of Obstetrics and Gynaecology Department, Dhaka, Bangladesh. Vaginal candidiasis [53.6% (134/250)] was the most common, followed by bacterial vaginosis [29.2% (73/250)], trichomoniasis [10.8% (27/250)] and gonorrhoea [1.2% (3/250)] (Yusuf *et al*., 2011).

Nevertheless, the results obtained in this study is in agreement with other studies, which showed that in 10% to 58% of the patients complaining of vaginal discharge, diagnosis could not be reached regardless of the diagnostic method under consideration (Rekha and Jyothi 2010). Pépin *et al.* (2005) found no pathogen responsible for VDS in 31.1% (258/826) of

female sex workers (FSW) recruited at the FSW/STI clinics in Accra (Ghana), Cotoñou and Porto Novo (Bénin). The prevalence of *M. genitalium* infection was 26.3% (217/826), compared to 23.1% (191/826) for *T. vaginalis*, 16.0% (132/826) for *N. gonorrhoeae* and 3.4% (28/826) for *C. trachomatis* using a commercial PCR assay (Amplicor, Roche Diagnostics, Branchburg, NJ, USA) for detection of *C. trachomatis* and *N. gonorrhoeae* and an in-house PCR assay was used for the detection of *M. genitalium* and *T. vaginalis*.

Zimba *et al*. (2011) did not detect pathogens in 22.1% (34/154) cases of VDS using culture and two molecular methods. Becton Dickinson Probetec Assays (Sparks, Maryland, USA) were performed for the detection of *C. trachomatis* and *N. gonorrhoeae* while an in-house conventional PCR assay was used for the detection of *M. genitalium* and *T. vaginalis*. *Neisseria gonorrhoeae* was diagnosed in 11% (17/154) of the patients; *C. trachomatis* was detected in 7% (10/154) of the patients and *T. vaginalis* in 2% (3/154) of the patients in a study conducted in Maputo, Mozambique. Rekha and Jyothi (2010) could not find pathogens in 28% of 146 cases of VDS. Finally, *C. trachomatis*, *M. genitalium*, *N. gonorrhoeae* and *T. vaginalis* are equally an important course of this syndrome as these pathogens are consistently diagnosed in varying rates regardless of population or laboratory method. The presence of one or more of these pathogens is strongly linked to VDS, but it is not exclusive.

In this study urethritis was diagnosed in 77% (48/62) of male participants; gonococcal urethritis was detected in 48% (30/62) cases, NGU in 29% (18/62) cases. Mixed gonococcal and NGU was diagnosed in 11% (7/62) of cases and no infection was diagnosed in 22% (14/62) of the participants. The high rate of gonococcal urethritis observed in this study is not peculiar, *N. gonorrhoeae* is often the most common cause of this condition (Low *et al*., 2014). In the study of Amin *et al.* (2007) approximately 73% of the studied men with acute urethritis could be attributed to gonococcal infection. Similarly, Al-Sweih *et al.* (2011) examined 426 symptomatic men attending eight government hospitals in Kuwait; 34.8% (77/426) positive gonococcal and 18.1% (40/426) chlamydia cases were identified using commercially available assays that simultaneously detected *C. trachomatis* and *N. gonorrhoeae*. Mhlongo *et al*. (2010) studied 290 and 217 patients with symptoms suggestive of urethritis in two South African cities (Cape Town and Johannesburg), 85.2% (247/290) and 71% (154/217) respectively were diagnosed as gonococcal urethritis (defined by real-time PCR assay).

In contrast, Ginocchio *et al*. (2012) reported only 1.7% (129/7579) gonococcal infection in 7 579 patients studied in the United States of America (USA) using the Aptima nucleic acid

amplification assay (Gen-Probe Incoporated, USA). Similarly Alli *et al*. (2011) reported a low rate of gonococcal urethritis [5% (5/100)] in a STI clinic in Ibadan, South-western Nigeria using culture and conventional biochemical tests. The differences in gonococcal rates can be attributed to the differences in the diagnostic method used, population sampled, number of participants sampled and the social and sexual behaviour of patients.

Non-gonococcal urethritis was diagnosed in 29% (18/62) of the male participants. *Chlamydia trachomatis*, *M. genitalium* and *T. vaginalis* each caused approximately 10% of the condition. This result is similar to the results obtained in the study of Le Roux *et al*. (2010) performed in Pretoria, South Africa using TMA, in which *C. trachomatis*, *M. genitalium* and *T. vaginalis* were the causative agents in 12.3% (38/300), 17.3% (52/300) and 8.0% (24/300) cases of NGU respectively among symptomatic men attending a family practice.

Mixed gonococcal and NGU was diagnosed in 11% (7/62) cases in this study. This consisted of 8% (5/62) *C. trachomatis* mixed with *N. gonorrhoeae* and 3% (2/62) *N. gonorrhoeae* mixed with *T. vaginalis*. No mixed infection was observed with *M. genitalium* and any other pathogen under consideration in all the male participants. These results are consistent with the study of Jensen *et al.* (2003) that reported no mixed infection between *C. trachomatis* and *M. genitalium* among the 73 urethral specimens studied in Denmark. Furthermore, *C. trachomatis* caused 13.7% (10/73) while *M. genitalium* caused 9.6% (7/73) cases of NGU reported by a PCR assay (Jensen *et al*., 2003). In the study by Le Roux *et al*. (2010), the most common mixed infection was with *N. gonorrhoeae* and *M. genitalium* [3.7% (11/300)], followed by *N. gonorrhoeae* and *C. trachomatis* [2.0% (6/300)].

The finding of mixed infections with *N. gonorrhoeae* and NGU pathogens is consistent with results obtained in the current study and it is not uncommon. Shafer *et al*. (2003) and Gaydos *et al*. (2006) reported *N. gonorrhoeae* mixed with NGU pathogens. In these two studies, *C. trachomatis* mixed infection with *N. gonorrhoeae* was reported as 11.6% and 30% respectively (Shafer *et al*., 2003; Gaydos *et al*., 2006). These data sets support both the European and CDC STD Treatment Guidelines, which recommends the co-treatment of chlamydia in men with gonorrhoea because of the frequency of coexistence of these pathogens (CDC, 2012).

Co-infection of urethritis and VDS pathogens (*C. trachomatis*, *M. genitalium, N. gonorrhoeae* and *T. vaginalis*) with HIV-1 was observed in the 100 participants enrolled at the antiretroviral clinic. Overall, 41% (41/100) of the participants had at least one of the VDS

and urethritis pathogens co-existing with HIV-1, while no pathogen was diagnosed in 59% (59/100) of the participants. The prevalence of MUS and VDS pathogens was 15% (15/100), 11% (11/100), 8% (8/100) and 7% (7/100) for *M. genitalium, C. trachomatis*, *N. gonorrhoeae* and *T. vaginalis* respectively.

The finding of co-infection of urethritis and VDS pathogens with HIV-1 is in agreement with the study of Aboud *et al*. (2008) conducted in three countries (Malawi, Tanzania, Zambia) among 2 292 pregnant women enrolled in a HIV prevention trial where participants was tested for STI. The co-infection rate with HIV-1 reported was: 18.8% (430/2292) *T. vaginalis*, 2.6% (60/2 292) *C. trachomatis* and 1.7% (39/2 292) *N. gonorrhoeae*. Similarly, McCoy *et al*. (2009) in a study performed in North Carolina reported a 31% (23/75) co-infection rate of STIs with HIV-1; 13% (3/23) chlamydia, 39% (9/23) gonorrhoea and 22% (5/23) trichomoniasis. Diaz *et al*. (2009) found that 31% (453/1 462) of men who have sex with men (MSM) in Spain with HIV-1 had chlamydia or gonorrhoea.

The varying prevalence of the causative pathogens of MUS and VDS in these populations is not unexpected, since different diagnostic methods were used in the various studies. Interestingly, the prevalence of *M. genitalium* in the HIV positive participants was high; this could be as a result of *M. genitalium*'s failure to respond to the drug (doxycycline) used to treat non-gonococcal urethritis in MUS patients (Black *et al*., 2008). *Mycoplasma genitalium* is more prevalent than other nonviral STIs in a HIV-1 positive population (Mavedzenge *et al*., 2012). Mavedzenge *et al*. (2012), reported that, *M. genitalium* was more prevalent among the HIV-1 group (14.8%) than the control group (6.5%).

The overall sensitivity and specificity of the real-time multiplex PCR assay used in this study was 100% and 75% respectively for both urine and vaginal swabs when compared to gonococcal culture results for the detection of *N. gonorrhoeae*. In the study of Goire *et al*. (2011), the sensitivity and specificity of the PCR assay for the detection of *N. gonorrhoeae* was 100% and 98.7% compared to bacterial culture results. These results are consistent with Diemert *et al.* (2002) who reported that PCR have sensitivities ranging from 92.4% to 100% for urethral swabs and from 42.3% to 94.1% for urine specimens. Furthermore, Knox *et al*. (2002) reported sensitivities of 97.2%, 92.6% and 71.9% for the detection of *N gonorrhoeae* by PCR assay using self-collected tampons, endocervical swabs and self-collected swabs respectively. Finally, Chernesky *et al*. (2014) reported clinical sensitivities ranging from 63.6% to 100% and specificities ranging from 98.4 to 100% for *N. gonorrhoeae* in a study

that compared four second-generation nucleic acid amplification assays [Tigris system, Aptima Combo 2 assay (Hologic Gen-Probe), the RealTime CT/NG assay on an m2000 instrument (Abbott), the ProbeTec CT/GC Q^x assay on the Viper system (Becton Dickinson, Sparks, Maryland, USA) and Cobas CT/NG assay on the Cobas 4800 platform (Roche Molecular Diagnostics, Germany)] performed on self-collected vaginal swabs (SCVS) and first-void urines (FVU) from 575 women in Ontario, Canada.

The real-time multiplex PCR assay reported in this study is a highly sensitive and specific test for screening of MUS and VDS pathogens. The protocol is easy to implement especially in laboratories that have existing equipment (open platforms). Hence, this real-time multiplex PCR assay can offer alternatives to the range of commercial tests available. The use of non-invasive (self-collected urine and vaginal swab) specimens for the real-time multiplex PCR assay makes it desirable, because these specimens have the potential of providing a more convenient means of screening a large number of people for these important STIs. Testing of first-void urine samples by real-time multiplex PCR assay could facilitate improved understanding of the epidemiology of these important STIs in Tshwane, South Africa.

One of the limitations of this study was that an additional molecular assay was not used to confirm all positive and negative real-time multiplex PCR assay results. Secondly a quantitative real-time multiplex PCR assay was not performed to determine the limit of detection of the current real-time multiplex PCR assay, thus presenting a potential for false negative reporting. Finally, the sample size is too small to extrapolate the prevalence of MUS and VDS in the Tshwane region.

4.6 Conclusion

The optimized real-time multiplex PCR assay was a sensitive diagnostic assay, while maintaining high specificity for diagnosing *N. gonorrhoeae* in the study population. The method was validated and has worked well on both urine and vaginal swab specimens. Non-gonococcal urethritis caused by *T. vaginalis* was the most common type of infection identified in this study, mixed simultaneous gonococcal and NGU were also detected. Therefore, testing for more than one pathogen in a PCR assay could be a useful approach for the laboratory diagnosis of MUS and VDS. This study showed that the syndromic management approach currently results in more than 40% overtreatment for patients with symptomatic MUS and VDS in the Tshwane region.

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Table 4.1: Nucleotide sequences of the primers and probes used in the multiplex real-time PCR assay for the detection of *N. gonorrhoea, M. genitalium, C. trachomatis* and *T. vaginalis*

Specific target	Primer-Probe Sequence (5'→3')	Ref
papTM-F papTM-R papTM-P	CAG CAT TCA ATT TGT TCC GAG TC GAA CTGG TTT CAT CTG ATT ACT TTC CA FAM-CGCCTATACGCCTGCTACTTTCACGC- Zen Iowa Black	(Goire *et al*., 2008)
My-1S-F My-2A-R My-ins-F MGS-2-R Mgen-P	GAA Tag CCA CAA TGG GAC TGA GA TCA CGA CAC GAG CTG ACG ACA AC AGG TCG CAA GCG TTA TC CAC CAC CTG TCA CTC GGT TAA CCT C CAL610-CTGTCGGAGCGATCCCTTCCGG TA-Zen Iowa Black	(Yoshida *et al*., 2002) (Hooton *et al*., 1988)
CP-F CP-R CP-P	CATGAAAACTCGTTCCGAAATAGAA TCAGAGCTTTACCTAACAACGCATA CAL590 –TCGCATGCAAGATATCGA- Zen Iowa Black	(Jaton *et al*., 2006)
TV2-F TV2-R TV2-P	Fw: AAG ATG GGT GTT TTA AGC TAG ATA AGG T Rv: CGT CTT CAA GTA TGC CCC AGT AC Cy 5-CCGAAGTTCATGTCCTCTCCAAGCGT-Zen Iowa Black	(Ferandon *et al*., 2011)

Table 4.2: Different techniques used in the diagnosis of *N. gonorrhoeae* from all participant specimens

	Gram stain		Culture		PCR	
	N	(%)	N	(%)	N	(%)
Positive	38	19%	10	5%	57	28%
Negative	162	81%	190	95%	143	72%
Total	**200**	**100%**	**200**	**100%**	**200**	**100%**

N: number of isolates positive per test method

Table 4.3: Prevalence comparison between the two clinics, gender and the overall infection rate by the real-time multiplex PCR assay

Variables/ Pathogens	STI clinic (HIV negative) (N=100)%	(HIV positive) (N=100) %	Gender		Overall (N=200)
			Male(N=62)%	Female (N=138)%	
C. trachomatis	8	11	10	9	9.5
M. genitalium	-	15	10	7	7.5
N. gonorrhoeae	49	8	48	20	28.5
T. vaginalis	15	7	10	12	11
Total	**72%**	**41%**	**78%**	**48%**	**56.5**

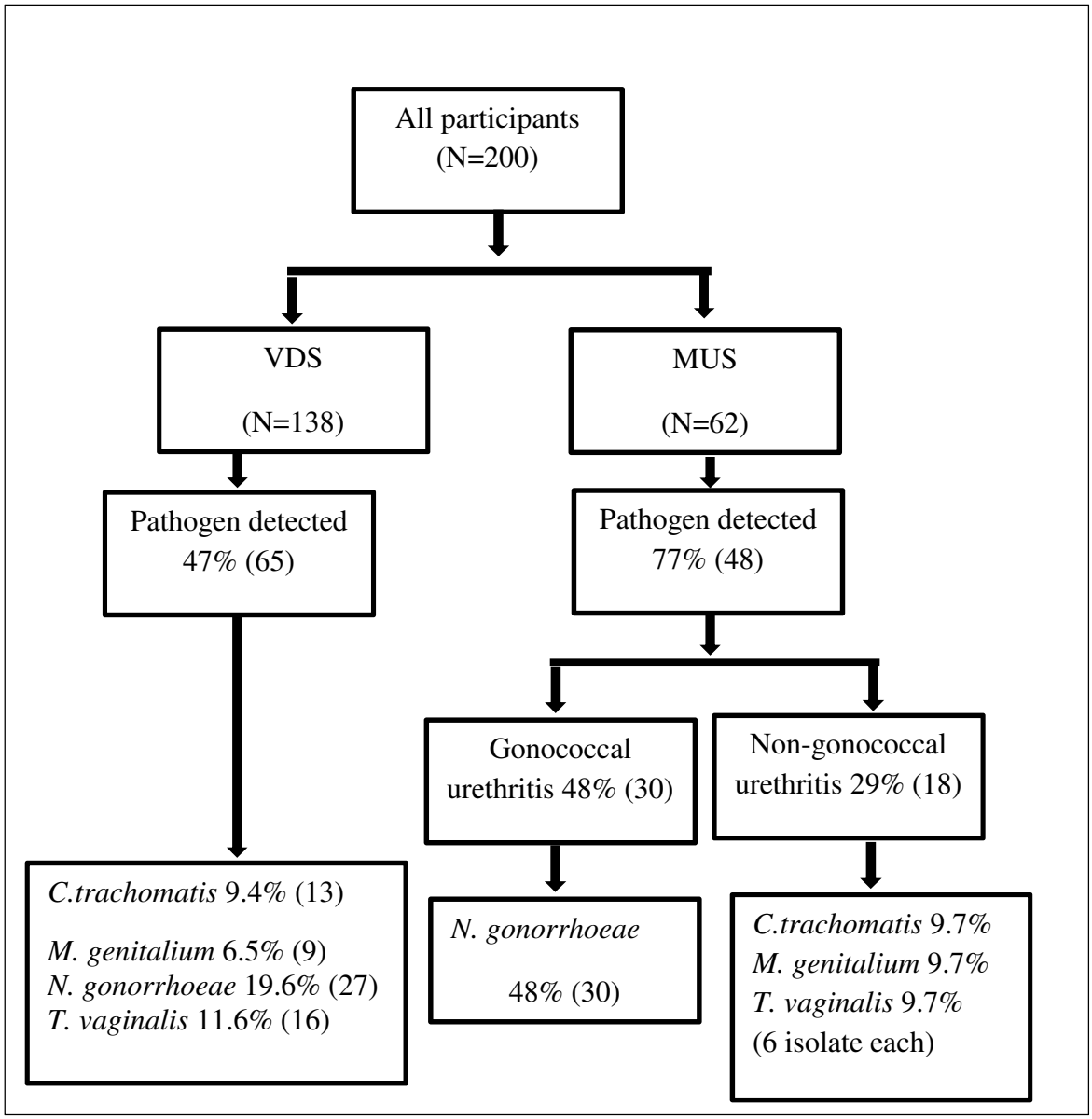

Figure 4.1: **Pathogen detection according to symptoms**

CHAPTER 5

Concluding remarks

5.1 Conclusions

Sexually transmitted infections (STIs) are a major health problem worldwide, especially in developing countries (Al-Sweih *et al*., 2011). Untreated STIs, particularly gonococcal and chlamydial infections can cause pelvic inflammatory disease in women and lead to infertility in both men and women (Muvunyia *et al*., 2011). Other complications, such as ectopic pregnancy and risk of developing ascending infection that leads to acute salpingitis may also develop from untreated STIs (Miller, 2006; Muvunyia *et al*., 2011). As a result accurate clinical and laboratory data about the prevalence of STIs is vital.

Infections caused by *N. gonorrhoeae* are one of the most widely distributed STIs worldwide (Cao *et al*., 2008; Unemo and Shafer, 2011). Hence early detection and appropriate antimicrobial treatment is needed in order to eliminate the pathogen (Endimiani *et al*., 2014). The challenge, however, is the constant change in the antimicrobial resistance of *N. gonorrhoeae* as a result of the extensive use and/or misuse of antibiotics for the treatment of gonorrhoea and the importation of resistant strains (Cao *et al*., 2008; Endimiani *et al*., 2014). Currently, extended-spectrum cephalosporins (ESCs) (ceftriaxone and cefixime) are used as the first-line treatment of gonococcal infections (Allen *et al*., 2011; Walker and Sweet, 2011), but the emergence and spread of cephalosporin resistant *N. gonorrhoeae* threatens the effectiveness of this regimen and poses a major public health challenge (Chesson *et al*., 2014). In the past, this was not a problem as alternative antibiotics existed, however, now the list of available treatments is diminishing quickly (Spiteri *et al*., 2013).

The antimicrobial susceptibility (AMS) pattern of bacterial pathogens changes constantly over time (Cao *et al*., 2008). This is true from the data obtained from this study, which suggested that the AMS pattern of *N. gonorrhoeae* in Tshwane, South Africa is changing. The *N. gonorrhoeae* isolates in this study were resistant to all first-line antimicrobial agents previously used in the treatment of gonococcal infections (fluoroquinolone, penicillin and tetracycline). An increase in the prevalence of *N. gonorrhoeae* isolates resistant to these antibiotics was observed in comparison with earlier studies in the region (De Jongh *et al*., 2007; Dangor *et al*., 2010). A dramatic increase in the MIC of these antibiotics was also

observed even though none of these agents are still in use as the first-line treatment of gonococcal infections. Additionally, the occurrence of isolates simultaneously resistant to ciprofloxacin, penicillin and tetracycline was high (50%). Hence, as the clinical strains move towards resistance to newer antibiotics, such as cephalosporin, it is unlikely that these antibiotics (ciprofloxacin, penicillin and tetracycline) will ever again become effective against gonorrhoea (Tomberg *et al*., 2013).

All isolates in this study were fully susceptible to cefixime, an antimicrobial agent that is currently in use as the first-line antimicrobial agent for the treatment of gonococcal infections (Lewis, 2011). It is noteworthy that this antibiotic (cefixime) has been excluded from the first-line empirical antimicrobial treatment recommendations in Europe, Japan and the United States of America (USA) (JSSTI, 2011; CDC, 2012; Bignell and Unemo, 2013). This is in response to reports of treatment failures and increasing MICs to this antibiotic (Chen *et al.,* 2014). In view of these differences in AMS patterns between countries, it is crucial to generate valid and quality-assured AMS data for gonococci to enhance local treatment guidelines. The present study recommends the need for a surveillance system that will continuously monitor the AMS patterns of *N. gonorrhoeae* in the Tshwane region for determination of optimal treatment regimens.

The prevalence of multidrug-resistant (MDR) *N. gonorrhoeae* isolates was high [50% (5/10)] in this study. This phenomenon is not new and has been observed in other countries (Allen *et al*., 2011; Unemo and Nicolas, 2012). This could have resulted from the extensive use of antibiotics for gonococcal treatment without performing the antimicrobial susceptibility testing (Endimiani *et al*., 2012). The indiscriminate use of antibiotics has generated not only an escalation of "treatment failures" (Unemo *et al*., 2010; Ison *et al*., 2011; Lewis *et al*., 2013), but also a selective pressure for specific successful international MDR clones, such as *N. gonorrhoeae* multi-antigen sequence type (NG-MAST), ST 1407 and ST 225 (Ison and Alexander, 2011; Unemo and Nicolas, 2012). For these same reasons, the NG-MAST belonging to ST 4822 with a MDR phenotype identified in this study might be a successful local clone, as it has been identified for the second time in the Gauteng province.

The isolation of ST 4822 in the public healthcare setting in this study is a cause for alarm, because specimens are rarely sent to public sector laboratories for testing as a result of implementation of the syndromic management approach (Lewis *et al*., 2013). Sequence type 4822 has a MDR phenotype and has been previously associated with cefixime treatment

failure in Johannesburg, South Africa (Lewis *et al*., 2013). It is, therefore, safe to speculate that this *N. gonorrhoeae* sequence type is widespread within the Gauteng province. Hence, caution for recurrent infections, treatment failures and laboratory testing of clinical specimens, is essential for appropriate patient management (Lewis *et al*., 2013).

The rate of detection of the aetiological pathogens of male urethritis syndrome (MUS) and vaginal discharge syndrome (VDS) (*Chlamydia trachomatis, Mycoplama genitalium, Neisseria gonorrhoeae* and *Trichomonas vaginalis*) in this study was high (56.5%). This infection rate by far cannot be used as the prevalence rate of STIs in the Tshwane region for the following reasons. The two healthcare facilities included in this study are visited by high risk individuals, sampling in these settings can thus lead to over-reporting of the prevalence of these STIs. Asymptomatic carriage of STIs (Newman *et al.,* 2007; Tsai and Li, 2013) was not considered in this study, this single variable can lead to under-reporting of the prevalence of these STIs in the region. Finally factors, such as the lack of awareness, cultural and economic barriers and the mindset of women to hide the symptoms until the development of serious complications, can reduce the attendance of infected women to healthcare facilities, hence, affecting prevalence reporting (Shrivastava *et al*., 2014).

The use of molecular assays that detect more than one STI pathogen in a single test for the routine laboratory diagnosis of STIs is advocated in this study. The importance of the syndromic management approach in resource limited settings is acknowledged. The syndromic management approach treats a patient at first visit and has wide accessibility because of its availability at primary healthcare level (WHO, 2007; Shrivastava *et al*., 2014). The shortfalls of this STI management approach (lack of specificity, risk of overtreatment and inability to detect asymptomatic infection) have necessitated the need for evaluation of other rapid diagnostic strategies to improve STI management (Hawkes *et al*., 1999; Vuylsteke, 2004; Romoren *et al*., 2007). Romoren and colleagues (2007) reported that clinical screening for signs of VDS at the initial antenatal care visit were more effective than the mere use of symptoms as an entry point for metronidazole treatment in a study conducted in Botswane.

The real-time multiplex PCR assay described in this study was a sensitive and specific (100% and 75%) test for the detection of *N. gonorrhoeae* from urine and vaginal swab specimens compared to the culture method. The low specificity obtained by the real-time multiplex PCR assay in this study is as a result of the high number of negative culture results. Urine and self-collected vaginal swab specimens are not the ideal specimens for *N. gonorrhoeae* culture.

Nevertheless, the real-time multiplex PCR assay was able to detect an overall 29% (57/200) *N. gonorrhoeae,* 11% (22/200) *T. vaginalis*, 10% (19/200) *C. trachomatis* and 8% (15/200) *M. genitalium* from these same specimen types. This assay has the advantage of ease of use, cheap (cost per reaction), it can be an important tool in a screening programme as it has the ability to detect asymptomatic carriage of MUS and VDS pathogens.

The rate of infection with *C. trachomatis, M. genitalium N. gonorrhoeae* and *T. vaginalis* was disproportionate between the two populations and sexes in this study. In general, a higher infection rate [72% (72/100)] was observed from participants from the STI clinic compared to participants from the ARV clinic [41% (41/100)]. This observation could be an indication that the HIV-1 positive group is more health conscious than the STI clinic group due to counselling offered at the ARV clinic. Furthermore, the relatively low rate reported in this group could also be attributed to the antibiotics (ciprofloxacin plus doxycycline) included in the treatment regimen for HIV, which act against gonococcal and non-gonococcal urethritis.

5.2 Future research

The findings of this study support the continued investigation of affordable, rapid and accurate diagnostic tests for the detection of STI. A large study on the sensitivity and specificity of the latest multiplex real-time PCR assay is required. Furthermore, expansion of the STI panel to include viral courses of urethral and vaginal discharge syndromes should be investigated. A rapid point of care nucleic acid amplification test (NAAT assay), that can be performed at on-site laboratories or in doctor's room maybe an ideal test to guide therapeutic decision making. Gaydos *et al*. (2013) described the Cepheid GeneXpert CT/NG (Xpert) as one of such tests.

Historically, the mainstay of *N. gonorrhoeae* antimicrobial surveillance was based on culture diagnosis and antimicrobial susceptibility testing. However, even under optimal sample handling and laboratory conditions, the sensitivity of bacterial culture remains poor for samples that have been taken from anatomical sites where the organism load is low (Goire *et al*., 2014). Non-culture-based antimicrobial surveillance may be possible using the *N. gonorrhoeae* (NG–MAST) system. Another non-culture antimicrobial susceptibility testing method could be the optimization of rapid molecular tests able to provide adequate information to select the most appropriate direct antibiotic treatment.

Optimisation of a multiplex real-time PCR that targets resistance determinant genes in *N. gonorrhoeae* is an option that should be explored. Such a PCR assay will have the advantage of rapid screening of these genes and aid surveillance programmes. The deployment of next-generation DNA-sequencing technologies has greatly enhanced capabilities for sequencing large meta–datasets (Petrosino *et al*., 2009). The use of these technologies has emerged as a cost-effective and convenient approach for addressing many microbiological questions (Didelot *et al*., 2012). Future STI research should focus on taking advantage of these next-generation DNA-sequencing technologies. Further investigation on novel antimicrobial families may be a way forward in preparation of total resistance to the cephalosporins. As we prepare for a future with untreatable gonococcal infections, efforts should be made to reduce the indiscriminate use of antibiotics, improve antimicrobial surveillance and embrace technologies that are aimed at improving the detection of STIs.

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APPENDIX A

Buffers, gels and reagents used in experimental procedures

1. Ethylene diamine tetra-acetate (EDTA) (0.5 M) (1 L) (pH 8.0)

EDTA, disodium salt (Merck, Germany)	186.1	g
Distilled water	800	ml
Sodium hydroxide (NaOH) pellets (Merck, Germany)		

Dissolve 186.1 g of EDTA in 800 ml of distilled water and mix until the solution becomes clear. Use the NaOH pellets to adjust the pH to 8.0 and bring the volume to 1 L, autoclave at 121ºC for 15 min.

2. Tris-boric EDTA (TBE) buffer 5X (pH 8.2) (1 L)

Tris-base (Sigma-Aldrich, USA)	54.0	g
Boric acid (Merck, Germany)	27.5	g
0.5 M EDTA (pH 8.0)	20	ml
Distilled water	800	ml

Dissolve 54 g of Tris-base and 27.5 g of boric acid in 800 ml of distilled water and add 20 ml of 0.5 M EDTA buffer. Adjust the pH to 8.2 and bring the volume to 1 L, autoclave at 121ºC for 15 min. A 1X TBE working solution was prepared by adding 200 ml of 1X TBE to 800 ml of distilled water.

3. Agarose gels

Low Electroendosmosis (LE) agarose gel

SeaKem LE agarose powder (Lonza, USA)	1	g
TBE buffer (1X)	100	ml
Ethidium bromide (10 mg/ml) (Promega, USA)	5	µl

Add 1 g of SeaKem LE agarose powder in 100 ml of 1X TBE [45 mM Tris-borate (pH 8.0) (Sigma Chemical co, USA), 0.5 M EDTA (Promega, Madison, USA)] and mix by swirling. Dissolve by heating in a microwave for 2 to 3 min. Cool down at 50ºC in a Hybridiser HB-1D oven (Techne, USA) set at 50ºC for at least 30 min. Add 5 µl of ethidium bromide (10 mg/ml) (Promega, USA) to the gel solution, mix gently and pour in a clean prepared casting tray. After the gel has set, the comb is removed to expose the wells for loading of DNA.

4. Trypticase soy broth (TSB) (1 L)

TSB powder (Oxoid, England)	30	g
Distilled water	1	L

Dissolve 30 g of TSB broth in 1 L of distilled water, autoclave at 121℃ for 15 min.

Distilled water	1	L

APPENDIX B

Experimental procedures

1. Deoxyribonucleic acid (DNA) extraction

1. Bacterial DNA was isolated with the ZR Fungal/Bacterial DNA kit (Zymo Research, USA) from the Copan E-swab in Amins transport medium (Copan Diagnostics, Inc, Italy)
2. One hundred and fifty microlitres of the Amies transport medium was added to a ZR BashingBead Lysis Tube (Zymo Research, USA) together with 750 µl Lysis solution.
3. The tube was vortexed (VELP Scientifica, Italy) for ± 5 min at maximum speed.
4. The ZR BashingBead Lysis Tube (Zymo Research, USA) was centrifuged in a Spectrafuge centrifuge (Labnet international, USA) at ≥10 000 *x g* for 1 min.
5. A volume of 400 µl of the supernatant was transferred to a Zymo-spin IV Spin Filter in a collection tube and centrifuged (Spectrafuge, Labnet International, USA) at 4 500 *x g* for 1 min.
6. A volume of 1 200 µl of bacterial DNA binding buffer was added to the filtrate in the collection tube,
7. A volume of 800 µl was transferred to a Zymo-Spin IIC column (Zymo Research, USA) in a collection tube and centrifuged (Spectrafuge, Labnet International, USA) at 10 000 *x g* for 1 min.
8. The flow-through from the collection tube was discarded and the latter step repeated.
9. Two hundred microlitres of DNA Pre-Wash Buffer (Zymo Research, USA) was added to the Zymo-spin IIC column (Zymo Research, USA) in a new collection tube and centrifuged (Spectrafuge, Labnet International, USA) at 10 000 *x g* for 1 min.
10. A volume of 500 µl of bacterial DNA wash buffer was added to the Zymo-spin IIC column (Zymo Research, USA) and centrifuged (Spectrafuge, Labnet International, USA) at 10 000 *x g* for 1 min.
11. The Zymo-spin IIC column (Zymo Research, USA) was transferred to a clean 1.5 ml microcentrifuge tube and 100 µl DNA elution buffer (Zymo Research, USA) was added to the column matrix and centrifuged (Spectrafuge, Labnet international, USA) at 10 000 *x g* for 30 s to elute the DNA.
12. The eluted ultra-pure DNA was stored at -20ºC until further analysis.

2. **Gram-staining of the vaginal swabs and the urine specimens for the detection of *Neisseria gonorrhoeae***

1. Smear onto a slide a thin layer of a single bacterial colony, fix by heating for 5 s using a Bunsen burner.
2. Gram staining was performed by flooding the heat-fixed smear with crystal violet (Diagnostic Media Products, NHLS, South Africa) for 1 min.
3. Excess crystal violet was rinsed off with water for 10 s.
4. Iodine solution (Diagnostic Media Products, NHLS, South Africa) was added for 1 min.
5. Excess iodine was rinsed off with water for 10 s.
6. Decolourisation with ethanol (Merck, Germany) was done for ± 30 s.
7. Excess ethanol was rinsed off with water for 10 s.
8. Bacterial cells were counterstained with safranin (Diagnostic Media Products, NHLS, South Africa) for 1 min.
9. Excess safranin was rinsed off with water for 10 s.
10. Gram-stained slides were dried with blotting paper.
11. Gram-stained slides were examined with a microscope (1000x) (Zeiss, Germany) for different morphotypes: Gram-negative intracellular diplococcic within polymorphonuclear leukocytes (PMNL).

3. **Wet-mount microscopy for the detection of *Trichomonas vaginalis***

1. Immediately after collection, vaginal swabs were eluted into ≤0.5 ml of sterile phosphate-buffered saline (PBS) (Life Technologies, Inc. USA) at room temperature (25ºC) and a slide is prepared with a drop of the saline sample and a coverslip. The urine specimens was centrifuged (Labnet International Inc, NJ, USA) at 500 *x* g for 5 min to obtain a sediment. Sediment was placed into 500 µl PBS (Life Technologies, Inc. USA). A small drop of the PBS (Life Technologies, Inc. USA) was then placed on a slide, a coverslip is placed on top of the fluid and the slide was examined by light microscopy (Zeiss, Germany) (WHO, 2013).
2. The slide was read at 100× magnification to look for motile trichomonads. Confirmation of pear-shaped morphology, including visualization of flagella, is performed using 400 × magnifications.

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APPENDIX C

Patient information data

Table 1: STD clinic's female participants' data and results for diagnostic tests done

			Biochemical test		Multiplex Real-time PCR					
Sample ID	**Gram stain**	**Culture**	**Oxidase test**	**API/NH**	**CT**	**MG**	**NG**	**TV**	**IC C_T**	**Age**
VS001	Negative	Positive	Negative	HS	-	-	+	-	31.51	27
VS002	Positive	Negative	Negative	NT	-	-	+	-	31.11	51
VS003	Positive	Negative	Negative	NT	-	-	-	-	31.66	28
VS004	Positive	Negative	Negative	NT	-	-	-	-	31.07	32
VS005	Positive	Negative	Negative	NT	-	-	-	-	31.43	U
VS006	Negative	Positive	Negative	HS	-	-	+	-	31.6	19
VS007	Negative	Negative	Negative	NT	-	-	-	-	31.19	36
VS008	Negative	Negative	Negative	NT	-	-	-	-	30.91	34
VS009	Positive	Negative	Negative	NT	-	-	+	-	31.59	47
VS010	Positive	Negative	Negative	NT	-	-	-	-	31.02	25
VS011	Positive	Negative	Negative	NT	-	-	-	-	31.5	31
VS012	Positive	Negative	Negative	NT	-	-	+	-	31.56	23
VS013	Negative	Negative	Negative	NT	-	-	-	-	31.16	29
VS014	Negative	Negative	Negative	NT	-	-	-	-	30.98	42
VS015	Negative	Negative	Negative	NT	-	-	-	-	31.17	28
VS016	Negative	Negative	Negative	NT	-	-	+	-	31.04	42
VS017	Positive	Negative	Negative	NT	-	-	+	-	31.73	U
VS018	Positive	Negative	Negative	NT	-	-	-	-	31.07	39
VS019	Positive	Negative	Negative	NT	-	-	+	-	31.21	37
VS020	Positive	Negative	Negative	NT	-	-	-	+	31.63	67
VS021	Positive	Negative	Negative	NT	-	-	-	+	31.54	43
VS022	Positive	Negative	Negative	NT	-	-	+	+	31.37	36
VS023	Positive	Negative	Negative	NT	-	-	+	+	31.2	46

CT: *Chlamydia trachomatis* NG: *Neisseria gonorrhoeae* IC: Internal control C_T: Cycle threshold
MG *Mycoplama genitalium* TV: *Trichomonas vaginalis* HS: *Haemophilus* spp NT: Not tested U: Unknown

Table 1: STD clinic's female participants' data and results for diagnostic tests done (continued)

			Biochemical test		Multiplex Real-time PCR					
Sample ID	**Gram stain**	**Culture**	**Oxidase test**	**API/NH**	**CT**	**MG**	**NG**	**TV**	**IC C_T**	**Age**
VS024	Positive	Negative	Negative	NT	-	-	+	+	31.57	32
VS025	Negative	Positive	Positive	NG	-	-	+	+	31.50	20
VS026	Positive	Negative	Negative	NT	-	-	-	+	29.83	33
VS027	Positive	Negative	Negative	NT	-	-	+	+	29.32	37
VS028	Positive	Negative	Negative	NT	-	-	-	+	29.58	43
VS029	Negative	Negative	Negative	NT	-	-	-	-	29.42	31
VS030	Positive	Negative	Negative	NT	+	-	-	-	29.83	21
VS031	Positive	Negative	Negative	NT	-	-	-	-	29.64	31
VS032	Positive	Negative	Negative	NT	+	-	+	-	29.92	21
VS033	Positive	Negative	Negative	NT	-	-	-	-	30.15	24
VS034	Positive	Negative	Negative	NT	-	-	+	-	29.36	39
VS035	Positive	Negative	Negative	NT	-	-	+	-	29.6	32
VS036	Negative	Negative	Negative	NT	-	-	-	-	29.68	33
VS037	Positive	Negative	Negative	NT	-	-	+	-	29.68	35
VS038	Positive	Negative	Negative	NT	-	-	-	-	29.73	37
VS039	Positive	Negative	Negative	NT	-	-	-	-	28.68	32
VS040	Negative	Negative	Negative	NT	-	-	+	-	29.64	27
VS041	Negative	Negative	Negative	NT	-	-	-	-	29.5	38
VS042	Positive	Negative	Negative	NT	-	-	-	-	29.62	21
VS043	Positive	Negative	Negative	NT	-	-	-	-	29.5	39
VS044	Positive	Negative	Negative	NT	-	-	-	-	29.44	29
VS045	Positive	Negative	Negative	NT	-	-	-	-	29.64	36
VS046	Positive	Negative	Negative	NT	+	-	+	-	29.2	31
VS047	Positive	Negative	Negative	NT	-	-	+	-	29.28	32
VS048	Negative	Positive	Positive	NG	-	-	+	-	29.2	25
VS049	Positive	Negative	Negative	NT	-	-	+	-	29.2	31
VS050	Positive	Negative	Negative	NT	-	-	+	-	29.6	43

CT: *Chlamydia trachomatis* NG: *Neisseria gonorrhoeae* IC: Internal control C_T: Cycle threshold
MG *Mycoplama genitalium* TV: *Trichomonas vaginalis* HS: *Haemophilus* spp NT: Not tested U: Unknown

Table 2: STD clinic's male participants' data and results for diagnostic tests done

			Biochemical test		Multiplex Real-time PCR					
Sample ID	**Gram stain**	**Culture**	**Oxidase test**	**API/NH**	**CT**	**MG**	**NG**	**TV**	**IC C_T**	**Age**
MU001	Negative	Positive	Negative	HS	+	-	+	-	28.18	36
MU002	Positive	Negative	Negative	NT	-	-	+	-	28.96	35
MU003	Positive	Negative	Negative	NT	-	-	+	-	28.71	28
MU004	Positive	Negative	Negative	NT	-	-	+	-	29.51	29
MU005	Negative	Negative	Negative	NT	-	-	-	-	29.05	39
MS008	Positive	Negative	Negative	NT	-	-	+	-	29.51	27
MU007	Negative	Positive	Positive	NG	-	-	+	-	29.18	35
MS007	Negative	Positive	Positive	NG	-	-	+	-	28.18	35
MU009	Negative	Negative	Negative	NT	-	-	+	-	28.96	31
MU10	Negative	Negative	Negative	NT	+	-	+	-	28.78	27
MS10	Positive	Positive	Negative	HS	-	-	+	-	30.13	27
MU12	Positive	Negative	Negative	NT	-	-	+	-	29.1	53
MU13	Positive	Negative	Negative	NT	-	-	+	-	27.74	45
MU14	Negative	Negative	Negative	NT	+	-	+	-	30.13	36
MU15	Negative	Negative	Negative	NT	-	-	+	-	29.59	51
MU16	Positive	Negative	Negative	NT	-	-	+	-	29.35	26
MU17	Positive	Negative	Negative	NT	+	-	+	-	29.21	37
MU18	Positive	Negative	Negative	NT	-	-	-	+	29.52	27
MU11	Positive	Negative	Negative	NT	+	-	+	-	29.84	32
MU20	Positive	Negative	Negative	NT	-	-	-	-	28.79	31
MU21	Positive	Negative	Negative	NT	-	-	-	+	30.13	33
MU22	Positive	Negative	Negative	NT	-	-	+	-	29.19	62
MU23	Positive	Negative	Negative	NT	-	-	+	-	29.06	21
MU24	Negative	Positive	Positive	NG	-	-	-	-	29.21	60
MS24	Negative	Negative	Positive	NG	-	-	+	-	29.19	60

CT: *Chlamydia trachomatis* NG: *Neisseria gonorrhoeae* IC: Internal control C_T: Cycle threshold
MG *Mycoplama genitalium* TV: *Trichomonas vaginalis* HS: *Haemophilus* spp NT: Not tested U: Unknown

Table 2: STD clinic's male participants' data and results for diagnostic tests done (continued)

			Biochemical test		Multiplex Real-time PCR					
Sample ID	**Gram stain**	**Culture**	**Oxidase test**	**API/NH**	**CT**	**MG**	**NG**	**TV**	**IC C_T**	**Age**
MU26	Positive	Negative	Negative	NT	-	-	-	-	29.61	24
MU27	Positive	Negative	Negative	NT	-	-	-	+	32.64	25
MU28	Positive	Negative	Negative	NT	-	-	+	+	33.27	45
MU29	Positive	Negative	Negative	NT	-	-	-	+	31.31	22
MU30	Positive	Negative	Negative	NT	-	-	+	+	31.96	36
MU31	Positive	Negative	Negative	NT	-	-	+	-	32.64	33
MS31	Negative	Positive	Positive	NG	-	-	+	-	29.61	33
MU32	Positive	Negative	Negative	NT	-	-	+	-	29.28	38
MU34	Positive	Negative	Negative	NT	-	-	+	+	32.65	28
MU35	Positive	Negative	Negative	NT	-	-	-	-	29.61	43
MU36	Positive	Negative	Negative	NT	-	-	-	-	29.28	29
MU37	Positive	Negative	Negative	NT	-	-	-	-	29.38	34
MU38	Positive	Negative	Negative	NT	-	-	-	-	29.3	44
MU39	Positive	Negative	Negative	NT	-	-	-	-	29.05	28
MU40	Positive	Negative	Negative	NT	-	-	-	-	29.46	27
MU41	Positive	Negative	Negative	NT	-	-	-	-	29.48	45
MU42	Positive	Negative	Negative	NT	-	-	-	-	29.72	33
MU43	Positive	Negative	Negative	NT	-	-	-	-	29.14	40
MU44	Positive	Negative	Negative	NT	-	-	-	-	29.81	23
MU45	Positive	Negative	Negative	NT	-	-	-	-	29.65	38
MU46	Positive	Negative	Negative	NT	-	-	-	-	29.57	40
MU47	Positive	Negative	Negative	NT	-	-	-	-	29.2	33
MU48	Positive	Negative	Negative	NT	-	-	-	-	29.59	25
MU49	Positive	Negative	Negative	NT	-	-	-	-	29.25	34
MU50	Positive	Negative	Negative	NT	-	-	-	-	29.28	21

CT: *Chlamydia trachomatis* NG: *Neisseria gonorrhoeae* IC: Internal control C_T: Cycle threshold
MG *Mycoplama genitalium* TV: *Trichomonas vaginalis* HS: *Haemophilus* spp NT: Not tested U: Unknown

Table 3: Antiretroviral clinic's female participants' data and results for diagnostic tests done

			Biochemical test		Multiplex Real-time PCR					
Sample ID	**Gram stain**	**Culture**	**Oxidase test**	**API/NH**	**CT**	**MG**	**NG**	**TV**	**IC C_T**	**Age**
F1	Positive	Negative	Negative	NT	-	-	-	-	29.14	29
F2	Positive	Negative	Negative	NT	-	-	-	-	29.2	U
F3	Positive	Negative	Negative	NT	-	-	-	-	29.43	U
F4	Positive	Negative	Negative	NT	-	-	-	-	29.47	U
F5	Positive	Negative	Negative	NT	-	-	-	-	29.4	44
F6	Positive	Negative	Negative	NT	-	-	-	-	30.22	28
F7	Positive	Negative	Negative	NT	+	-	-	+	35	37
F8	Positive	Negative	Negative	NT	-	-	-	-	30.26	35
F9	Negative	Negative	Negative	NT	-	-	-	-	27.97	48
F10	Positive	Negative	Negative	NT	-	-	-	-	29.32	24
F11	Positive	Negative	Negative	NT	-	-	-	-	29.97	42
F12	Positive	Negative	Negative	NT	-	-	-	-	30.03	44
F13	Positive	Negative	Negative	NT	-	-	-	-	30.03	68
F14	Positive	Negative	Negative	NT	-	-	-	-	30.18	30
F15	Positive	Negative	Negative	NT	-	-	-	-	30.07	34
F16	Negative	Negative	Negative	NT	-	-	-	-	29.96	32
F17	Positive	Negative	Negative	NT	-	-	-	-	30.08	34
F18	Positive	Negative	Negative	NT	-	-	-	-	30.46	42
F19	Positive	Negative	Negative	NT	-	-	-	-	29.6	42
F20	Positive	Negative	Negative	NT	-	-	-	-	29.53	41
F21	Positive	Negative	Negative	NT	-	-	-	-	30.26	40
F22	Positive	Negative	Negative	NT	-	-	-	-	30.26	43
F23	Negative	Negative	Negative	NT	-	-	+	-	28.32	37
F24	Positive	Negative	Negative	NT	-	-	+	-	27.9	39
F25	Negative	Positive	Positive	NG	-	-	+	-	30.03	46
F26	Negative	Positive	Positive	NG	-	-	+	-	30.03	27
F27	Positive	Negative	Negative	NT	-	-	-	-	29.5	38

CT: *Chlamydia trachomatis* NG: *Neisseria gonorrhoeae* IC: Internal control C_T: Cycle threshold
MG *Mycoplama genitalium* TV: *Trichomonas vaginalis* HS: *Haemophilus* spp NT: Not tested U: Unknown

Table 3: Antiretroviral clinic's female participants' data and results for diagnostic tests done (continued)

			Biochemical test		Multiplex Real-time PCR					
Sample ID	**Gram stain**	**Culture**	**Oxidase test**	**API/NH**	**CT**	**MG**	**NG**	**TV**	**IC C_T**	**Age**
F28	Positive	Negative	Negative	NT	-	-	-	-	29.6	30
F29	Negative	Negative	Negative	NT	-	-	-	-	30.05	30
F30	Positive	Negative	Negative	NT	-	-	-	-	29.95	29
F31	Positive	Negative	Negative	NT	-	-	-	-	29.89	27
F32	Positive	Negative	Negative	NT	-	-	-	-	29.81	31
F33	Positive	Negative	Negative	NT	-	-	-	-	29.98	U
F34	Positive	Negative	Negative	NT	-	-	-	-	30.01	37
F35	Positive	Negative	Negative	NT	-	-	-	-	29.68	42
F36	Positive	Negative	Negative	NT	-	-	-	-	30.02	49
F37	Positive	Negative	Negative	NT	-	-	-	-	30.12	27
F38	Positive	Negative	Negative	NT	-	-	-	-	30.08	31
F39	Positive	Negative	Negative	NT	-	-	-	-	30.06	30
F40	Positive	Negative	Negative	NT	-	-	-	-	30.09	37
F41	Negative	Negative	Negative	NT	-	-	-	-	30.11	33
F42	Positive	Negative	Negative	NT	-	-	-	-	29.87	36
F43	Positive	Negative	Negative	NT	-	-	-	-	30.14	35
F44	Positive	Negative	Negative	NT	-	-	-	-	29.93	38
F45	Positive	Negative	Negative	NT	-	-	-	-	29.92	52
F46	Positive	Negative	Negative	NT	-	-	-	-	29.97	52
F47	Positive	Negative	Negative	NT	-	-	-	-	30.03	32
F48	Positive	Negative	Negative	NT	-	-	-	-	30	61
F49	Positive	Negative	Negative	NT	-	-	-	-	30.04	33
F50	Positive	Negative	Negative	NT	-	-	-	-	29.93	37
F51	Positive	Negative	Negative	NT	-	-	-	+	29.96	25
F52	Positive	Negative	Negative	NT	-	-	-	-	29.95	46
F53	Positive	Negative	Negative	NT	-	-	-	-	30	43
F54	Positive	Negative	Negative	NT	-	-	-	-	29.9	28

CT: *Chlamydia trachomatis* NG: *Neisseria gonorrhoeae* IC: Internal control C_T: Cycle threshold
MG *Mycoplama genitalium* TV: *Trichomonas vaginalis* HS: *Haemophilus* spp NT: Not tested U: Unknown

Table 3: Antiretroviral clinic's female participants' data and results for diagnostic tests done (continued)

			Biochemical test		Multiplex Real-time PCR					
Sample ID	**Gram stain**	**Culture**	**Oxidase test**	**API/NH**	**CT**	**MG**	**NG**	**TV**	**IC C_T**	**Age**
F55	Positive	Negative	Negative	NT	-	-	-	-	30.05	29
F56	Positive	Negative	Negative	NT	-	-	-	-	29.86	36
F57	Positive	Negative	Negative	NT	-	-	-	-	30	40
F58	Negative	Negative	Negative	NT	-	-	-	-	29.75	39
F59	Positive	Negative	Negative	NT	-	-	-	-	29.95	33
F60	Positive	Negative	Negative	NT	-	-	-	-	29.86	31
F61	Positive	Negative	Negative	NT	-	-	-	-	29.88	33
F62	Positive	Negative	Negative	NT	-	-	-	-	29.64	22
F63	Positive	Negative	Negative	NT	-	+	-	-	28.07	29
F64	Negative	Negative	Negative	NT	-	+	-	-	29.6	43
F65	Positive	Negative	Negative	NT	-	+	-	-	30.05	31
F66	Positive	Negative	Negative	NT	-	-	-	-	29.95	41
F67	Positive	Negative	Negative	NT	-	-	-	-	29.89	34
F68	Positive	Negative	Negative	NT	+	+	-	-	29.81	50
F69	Positive	Negative	Negative	NT	-	+	-	-	29.98	44
F70	Negative	Negative	Negative	NT	-	-	+	-	30.01	37
F71	Positive	Negative	Negative	NT	+	+	-	-	29.68	40
F72	Positive	Negative	Negative	NT	-	-	-	+	30.02	27
F73	Positive	Negative	Negative	NT	+	-	-	-	30.12	30
F74	Positive	Negative	Negative	NT	+	+	-	-	30.08	29
F75	Positive	Negative	Negative	NT	-	-	-	-	30.06	28
F76	Positive	Negative	Negative	NT	-	-	-	-	30.09	37
F77	Positive	Negative	Negative	NT	+	-	-	+	30.11	32
F78	Positive	Negative	Negative	NT	-	-	-	+	29.87	47
F79	Negative	Negative	Negative	NT	-	-	-	-	30.14	30
F80	Positive	Negative	Negative	NT	-	-	-	-	29.93	26
F81	Positive	Negative	Negative	NT	-	-	-	-	29.6	26

CT: *Chlamydia trachomatis* NG: *Neisseria gonorrhoeae* IC: Internal control C_T: Cycle threshold
MG *Mycoplama genitalium* TV: *Trichomonas vaginalis* HS: *Haemophilus* spp NT: Not tested U: Unknown

Table 3: Antiretroviral clinic's female participants' data and results for diagnostic tests done (continued)

			Biochemical test		Multiplex Real-time PCR					
Sample ID	**Gram stain**	**Culture**	**Oxidase test**	**API/NH**	**CT**	**MG**	**NG**	**TV**	**IC C_T**	**Age**
F82	Negative	Negative	Negative	NT	+	+	-	-	29.6	31
F83	Positive	Negative	Negative	NT	-	-	-	-	30.05	32
F84	Positive	Negative	Negative	NT	+	-	-	-	29.95	38
F85	Positive	Negative	Negative	NT	-	-	-	-	29.89	26
F86	Positive	Negative	Negative	NT	+	+	-	-	29.81	33
F87	Positive	Negative	Negative	NT	+	-	-	+	29.98	36
F88	Positive	Negative	Negative	NT	-	-	-	+	30.01	27

CT: *Chlamydia trachomatis* NG: *Neisseria gonorrhoeae* IC: Internal control C_T: Cycle threshold
MG *Mycoplama genitalium* TV: *Trichomonas vaginalis* HS: *Haemophilus* spp NT: Not tested U: Unknown

Table 4: Antiretroviral clinic's male participants, data and results for diagnostic tests done

			Biochemical test		Multiplex Real-time PCR					
Sample ID	**Gram stain**	**Culture**	**Oxidase test**	**API/NH**	**CT**	**MG**	**NG**	**TV**	**IC C_T**	**Age**
M1	Positive	Negative	Negative	NT	+	-	-	-	29.1	44
M2	Negative	Negative	Negative	NT	-	+	-	-	29.07	35
M3	Positive	Negative	Negative	NT	-	+	-	-	28.7	46
M4	Positive	Negative	Negative	NT	-	+	-	-	28.4	U
M5	Positive	Negative	Negative	NT	-	-	-	-	28.5	U
M6	Positive	Negative	Negative	NT	-	-	-	-	27.73	33
M7	Positive	Negative	Negative	NT	-	+	-	-	28.51	31
M8	Positive	Negative	Negative	NT	-	+	-	-	28.53	18
M9	Positive	Negative	Negative	NT	-	+	-	-	28.2	25
M10	Negative	Positive	Positive	NG	-	-	+	-	29.06	41
M11	Negative	Positive	Positive	NG	-	-	+	-	29.02	30
M12	Positive	Negative	Negative	NT	-	-	+	-	29.07	41

CT: *Chlamydia trachomatis* NG: *Neisseria gonorrhoeae* IC: Internal control C_T: Cycle threshold
MG *Mycoplama genitalium* TV: *Trichomonas vaginalis* HS: *Haemophilus* spp NT: Not tested U: Unknown

APPENDIX D

Amplification curves of the multiplex real-time PCR assay

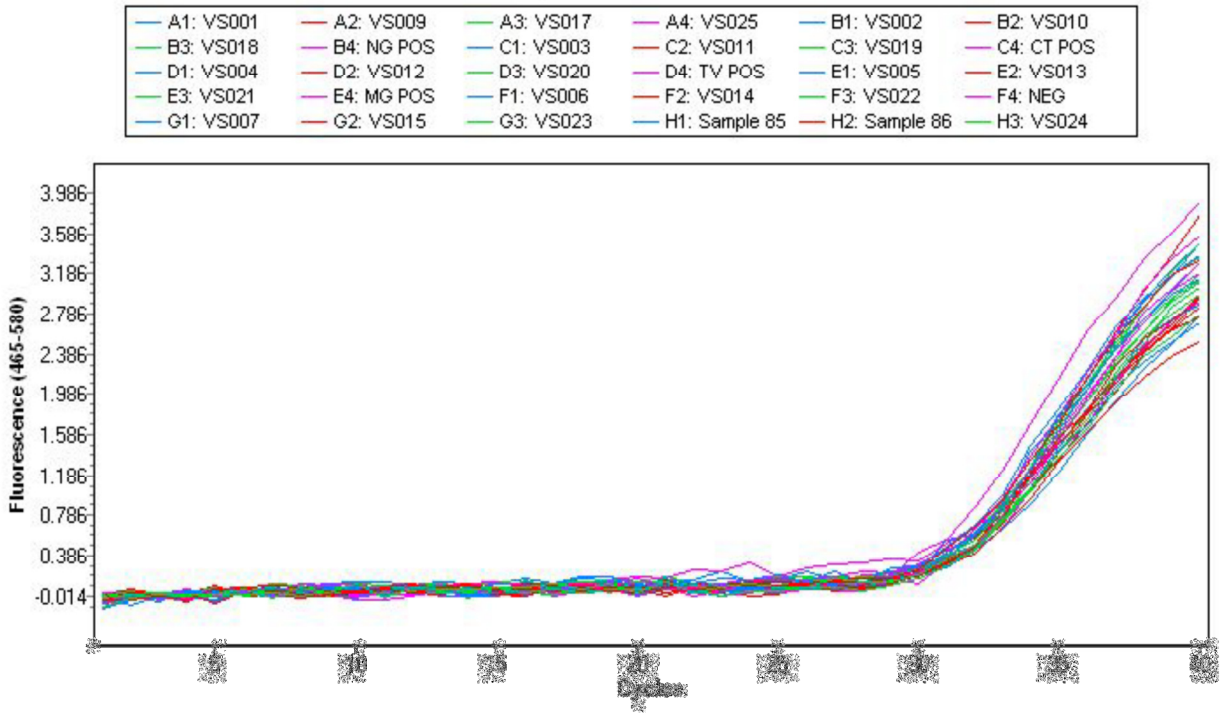

Figure 1: **The internal control amplification curve (detection in HEX channel 465 nm to 580 nm)**

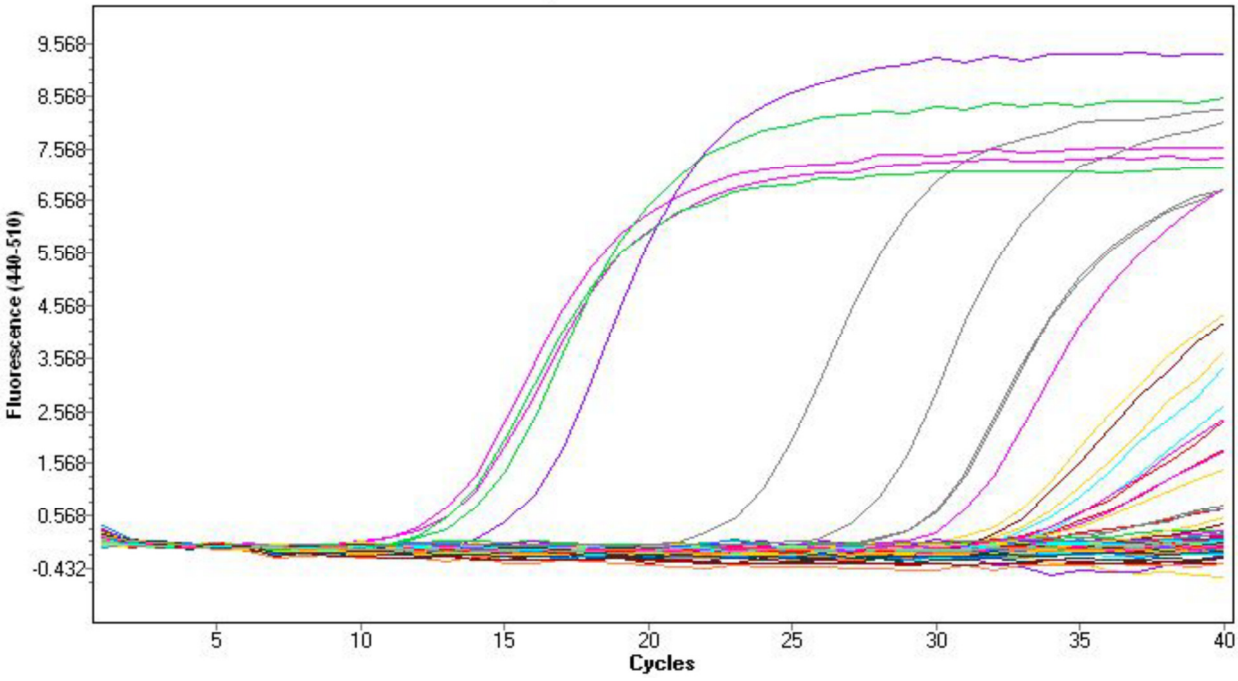

Figure 2: ***N. gonorrhoeae*** **amplification curve (detection in FAM channel 440 nm to 510 nm)**

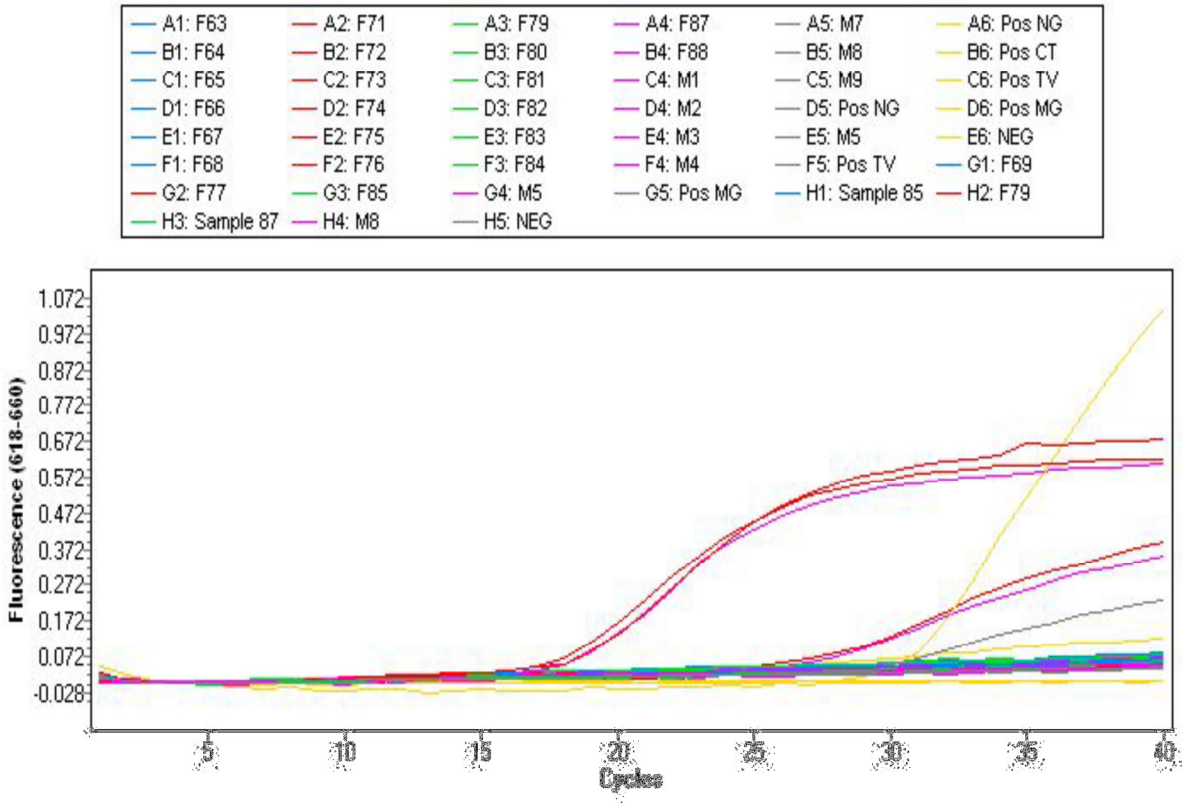

Figure 3: ***T. vaginalis*** **amplification curve (detection in Cy 5 618 nm to 660 nm)**

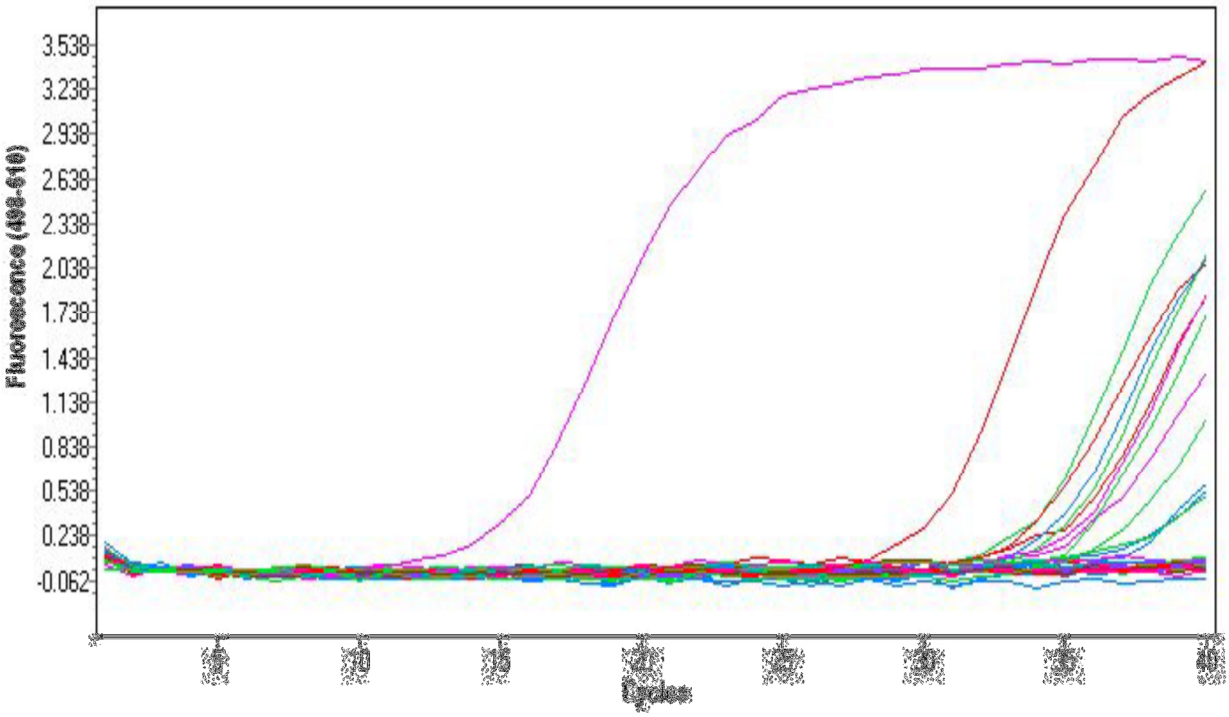

Figure 4: ***C. trachomatis*** **amplification curve (detection in CAL 590 channel 498 nm to 610 nm)**

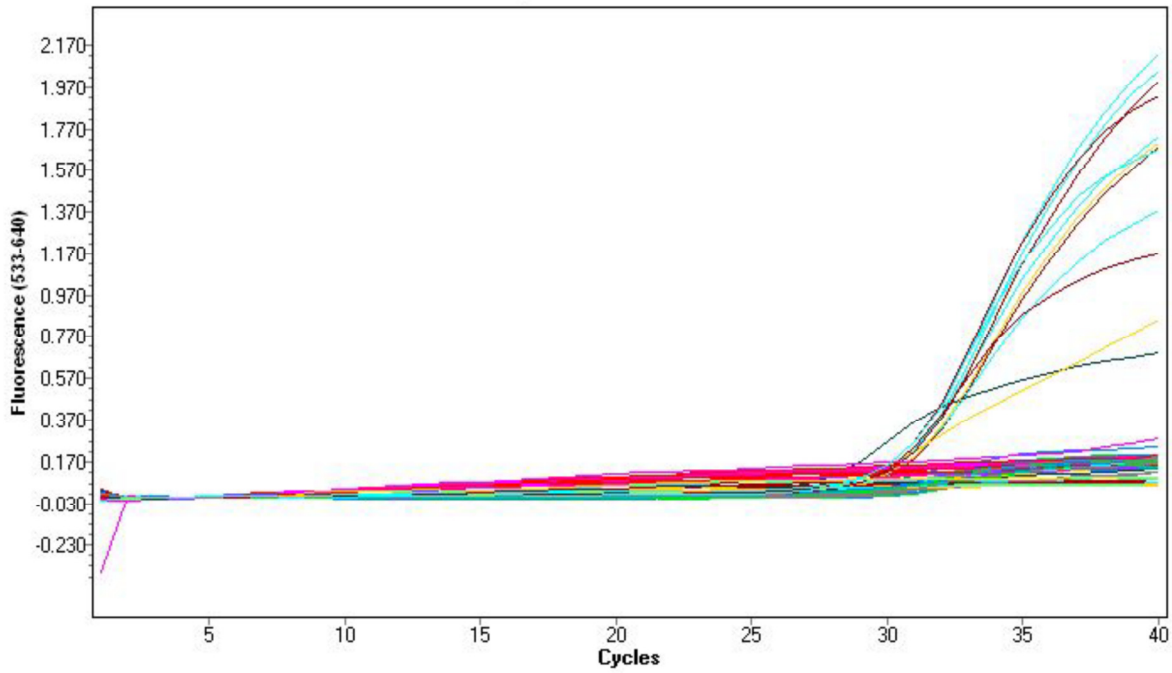

Figure 5: ***M. genitalium*** **amplification curve (detection in CAL 610 channel 533 nm to 640nm)**

APPENDIX E: Disc diffusion and E-test experiments raw data

Table 1: The AST results of culture positive isolates

Antibiotics	Patient: ATCC 19424					Patient: ATCC 31426					Patient: M 12				
	Test 1	Test 2	Test 3	Test 4	**Mean (mm)**	Test 1	Test 2	Test 3	Test 4	**Mean (mm)**	Test 1	Test 2	Test 3	Test 4	**Mean (mm)**
Penicillin 10 units	50	55	55	55	**53.75**	13	12	13	15	**13.25**	12	14	15	15	**14**
Cefoxitin 30 µg	40	37	40	40	**39.25**	40	42	45	40	**41.75**	33	35	35	35	**34.50**
Cefpodoxime 10 µg	55	52	55	55	**54.25**	50	55	50	52	**51.75**	43	45	45	45	**44.50**
Ceftriaxone 30 µg	50	52	50	51	**50.75**	50	52	52	55	**52.25**	48	48	50	50	**47**
Tetracycline 30 µg	35	38	40	40	**38.25**	38	40	44	42	**41**	25	25	25	27	**25.50**
Ciprofloxacin 5 µg	45	47	42	45	**44.75**	50	52	52	55	**52.25**	25	25	27	27	**26**
Spectinomycin 25 µg	11	10	10	13	**11**	15	15	17	17	**16**	12	12	10	12	**11.50**
Ofloxacin 5 µg	40	43	40	45	**42**	50	52	50	50	**50.50**	21	23	23	25	**23**
Cefixime 5 µg	50	50	55	52	**51.4**	55	50	50	52	**51.75**	43	43	40	43	**42.25**
Cefepime 30µg	60	57	57	58	**58**	60	60	60	58	**59.50**	55	57	55	55	**55.50**
Cefotaxime 30µg	62	63	60	60	**61.25**	60	60	50	55	**56.25**	45	45	47	45	**45.50**

Table 1: The AST results of culture positive isolates (continued)

Antibiotics	Patient: VS 048					Patient: MS 24					Patient: M 11				
	Test 1	Test 2	Test 3	Test 4	**Mean (mm)**	Test 1	Test 2	Test 3	Test 4	**Mean (mm)**	Test 1	Test 2	Test 3	Test 4	**Mean (mm)**
Penicillin 10 units	20	20	22	22	**21**	12	13	13	14	**13**	13	12	12	12	**12.25**
Cefoxitin 30 µg	34	33	35	35	**34.25**	35	35	37	35	**35.5**	25	27	25	25	**25.50**
Cefpodoxime 10 µg	40	35	35	35	**36.25**	50	48	54	50	**50.5**	40	42	41	40	**40.75**
Ceftriaxone 30 µg	50	47	47	45	**47.25**	50	52	58	55	**53.75**	48	46	46	47	**46.75**
Tetracycline 30 µg	13	15	15	16	**14.75**	20	22	22	22	**21.5**	22	20	20	20	**20.50**
Ciprofloxacin 5 µg	0	0	0	0	**0**	23	22	23	25	**23.25**	40	40	42	40	**40.50**
Spectinomycin 25 µg	10	7	7	6	**7.5**	12	13	13	14	**13**	20	18	18	18	**18.5**
Ofloxacin 5 µg	0	0	0	0	**0**	30	32	30	30	**30.5**	30	30	29	27	**29**
Cefixime 5 µg	35	37	35	37	**36**	40	45	48	45	**44.5**	45	45	47	46	**45.75**
Cefepime 30µg	35	35	33	34	**34.5**	25	25	25	27	**25.5**	60	60	58	58	**59**
Cefotaxime 30µg	40	40	42	43	**41.5**	50	55	55	55	**53.75**	45	45	47	45	**45.50**

Table 1: The AST results of culture positive isolates (continued)

Antibiotics	F 25					Patient: F 26					Patient: V 025				
	Test 1	Test 2	Test 3	Test 4	**Mean (mm)**	Test 1	Test 2	Test 3	Test 4	**Mean (mm)**	Test 1	Test 2	Test 3	Test 4	**Mean (mm)**
Penicillin 10 units	20	18	18	18	18.50	38	40	40	40	39.50	7	6	7	7	6.75
Cefoxitin 30 µg	30	30	30	30	30	40	40	40	38	39.50	34	34	33	32	33.50
Cefpodoxime 10 µg	40	38	38	40	39	52	52	53	53	52.50	40	40	40	40	40
Ceftriaxone 30 µg	25	30	26	25	39	55	55	56	55	55.25	45	43	43	43	43.50
Tetracycline 30 µg	25	25	25	25	25	30	29	28	28	28.75	0	0	0	0	0
Ciprofloxacin 5 µg	10	8	10	10	9.5	15	11	12	12	12.50	20	20	21	19	20
Spectinomycin 25 µg	12	10	12	12	11.50	20	10	12	12	54	13	12	12	11	12
Ofloxacin 5 µg	0	0	0	0	0	8	8	8	8	8	20	20	20	21	20.25
Cefixime 5 µg	50	50	50	48	49.50	50	52	52	53	51.75	40	40	40	40	40
Cefepime 30µg	40	40	42	40	40.50	55	55	55	57	55.50	41	42	40	40	40.75
Cefotaxime 30µg	60	60	60	60	60	55	57	57	57	56.50	47	45	45	45	45.50

Table 1: The AST results of culture positive isolates (continued)

Antibiotics	Patient: MS 007					Patient: MU 007					Patient: M 31				
	Test 1	Test 2	Test 3	Test 4	**Mean (mm)**	Test 1	Test 2	Test 3	Test 4	**Mean (mm)**	Test 1	Test 2	Test 3	Test 4	**Mean (mm)**
Penicillin 10 units	35	33	35	35	**34.50**	50	47	46	46	**47.25**	50	48	47	48	**48.25**
Cefoxitin 30 µg	37	37	36	35	**36.25**	40	40	38	40	**39.50**	45	42	43	44	**43.50**
Cefpodoxime 10 µg	40	42	42	42	**41.50**	55	58	55	55	**55.75**	70	68	68	68	**68.50**
Ceftriaxone 30 µg	50	50	52	52	**51**	60	57	57	60	**58.50**	70	69	68	69	**69**
Tetracycline 30 µg	30	28	28	30	**29**	10	8	8	9	**8.75**	8	8	8	6	**7.50**
Ciprofloxacin 5 µg	0	0	0	0	**0**	38	38	37	35	**37**	35	36	36	36	**35.75**
Spectinomycin 25 µg	15	13	12	12	**13**	30	28	28	30	**29**	25	23	23	24	**23.75**
Ofloxacin 5 µg	0	0	0	0	**0**	0	0	0	0	**0**	15	12	11	14	**13**
Cefixime 5 µg	40	37	38	40	**38.75**	65	62	62	63	**63**	70	70	68	70	**69.50**
Cefepime 30µg	38	38	36	36	**37**	65	65	65	63	**64.50**	70	70	68	68	**69**
Cefotaxime 30µg	50	48	51	48	**49.25**	60	60	62	60	**60.50**	70	67	66	68	**67.75**

Table 1: The MIC results of 10 isolates and two ATCC strains

Antibiotics	**Patient:** ATCC 19424				**Patient:** ATCC 31426				**Patient:** M 12			
	Test 1	Test 2	Test 3	Test 4	Test 1	Test 2	Test 3	Test 4	Test 1	Test 2	Test 3	Test 4
Penicillin 0.016-256µg/µl	0.023	0.023	0.023	0.023	12	12	16	16	3	3	3	3
Cefixime 0.016-25µg/µl	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.023	0.016
Cefpodoxime 0.016-25µg/µl	0.016	0.016	0.016	0.016	0.023	0.023	0.023	0.023	0.016	0.016	0.016	0.016
Cefotaxime 0.032-256 µg/µl	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016
Ciprofloxacin 0.002-3 µg/µl	0.03	0.03	0.04	0.04	0.004	0.003	0.004	0.004	0.50	0.50	0.50	0.50

Table 1: The MIC results of 10 isolates and two ATCC strains (continued)

Antibiotics	**Patient:** VS 048				**Patient:** MS 24				**Patient:** M 11			
	Test 1	Test 2	Test 3	Test 4	Test 1	Test 2	Test 3	Test 4	Test 1	Test 2	Test 3	Test 4
Penicillin 0.016-256µg/µl	1	0.5	1	1	24	24	24	24	5	5	7	5
Cefixime 0.016-25µg/µl	0.047	0.047	0.047	0.047	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016
Cefpodoxime 0.016-25µg/µl	2	2	2	2	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016
Cefotaxime 0.032-256 µg/µl	0.032	0.19	0.19	0.19	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016
Ciprofloxacin 0.002-3 µg/µl	0	0	0	0	0.50	0.50	0.50	0.50	3	4	4	3

Table 1: The MIC results of 10 isolates and two ATCC strains (continued)

Antibiotics	**Patient:** F 25				**Patient:** F26				**Patient:** VS 025			
	Test 1	Test 2	Test 3	Test 4	Test 1	Test 2	Test 3	Test 4	Test 1	Test 2	Test 3	Test 4
Penicillin 0.016-256µg/µl	3	3	3	3	0.19	0.125	0.125	0.125	16	16	16	16
Cefixime 0.016-25µg/µl	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016
Cefpodoxime 0.016-25µg/µl	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.047	0.047	0.047	0.047
Cefotaxime 0.032-256 µg/µl	0.75	0.75	0.75	075	0.016	0.016	0.016	0.016	0.023	0.023	0.023	0.023
Ciprofloxacin 0.002-3 µg/µl	2	2	2	2	3	3	3	4	1	1	1	1

Table 1: The MIC results of 10 isolates and two ATCC strains (continued)

Antibiotics	Patient: MS 007				Patient: MU 007				Patient: MS 31			
	Test 1	Test 2	Test 3	Test 4	Test 1	Test 2	Test 3	Test 4	Test 1	Test 2	Test 3	Test 4
Penicillin 0.016-256µg/µl	0.5	0.5	1	1	0.0125	0.0125	0.0125	0.0125	0.032	0.032	0.032	0.032
Cefixime 0.016-25µg/µl	0.047	0.047	0.047	0.047	0.047	0.047	0.047	0.047	0	0	0	0
Cefpodoxime 0.016-25µg/µl	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016
Cefotaxime 0.032-256 µg/µl	0.125	0.125	0.125	0.125	0.125	0.125	0.0125	0.0125	0.016	0.016	0.016	0.016
Ciprofloxacin 0.002-3 µg/µl	0	0	0	0	0.004	0.004	0.004	0.004	0.19	0.19	0.19	0.19