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Trichomonas vaginalis and bacterial co-infections identified in reproductive age women

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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 herein is my own original work and has not previously, in its entirety or in part, been submitted to any university for a degree. I further declare that all sources cited are acknowledged by means of a list of references.

Signed _____this _____day of _____ 2017

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LIST OF SYMBOLS AND ABBREVIATIONS

SYMBOLS

°C	-	Degree Celsius
µL	-	Microlitre

ABBREVIATIONS

2-D	-	Two-dimensional
AMR	-	Antimicrobial resistance
ATP	-	Adenosine triphosphate
AZT	-	Azithromycin
BV	-	Bacterial vaginosis
CDC	-	Centers for Disease Control and Prevention
CDS	-	Coding sequence
CEACAM	-	Carcino embryonic antigen-related cell adhesion molecule
CFX	-	Cefixime
CI	-	Confidence interval
CIP	-	Ciprofloxacin
CLIA	-	Clinical Laboratory Improvement Amendments
CLSI	-	Clinical and Laboratory Standards Institute
COR	-	Crude odds ratio
CP	-	Cysteine proteinase
CRO	-	Ceftriaxone
DNA	-	Deoxyribonucleic acid
EB	-	Elementary body
ECM	-	Extracellular matrix
ELISA	-	Enzyme-linked immunosorbent assay

ESC	-	Extended-spectrum cephalosporin
EUCAST	-	European Committee on Antimicrobial Susceptibility Testing
FDA	-	Food and Drug Administration
FISH	-	Fluorescence *in situ* hybridisation
Fn	-	Fibronectin
GC	-	Gonococcus
Hb	-	Haemoglobin
HIV	-	Human immunodeficiency virus
HSV-2	-	Herpes simplex virus-type 2
IVF	-	Intermediate vaginal flora
LGV	-	Lymphogranuloma venereum
LOS	-	Lipooligosaccharide
LPS	-	Lipopolysaccharide
MAbs	-	Monoclonal antibodies
MDR	-	Multi drug resistance
MIC	-	Minimum inhibitory concentration
MLST	-	Multilocus sequence typing
MLVA	-	Multilocus variable-number analysis
MOMP	-	Major outer membrane protein
M-PCR	-	Multiplex polymerase chain reaction
MtrCDE	-	Multiple transferable resistance *CDE*
MUDS	-	Male urethral discharge syndrome
MUS	-	Male urethritis syndrome
NAAT	-	Nucleic acid amplification test
NADPH	-	Nicotinamide adenine dinucleotide phosphate
NAH	-	Nucleic acid hybridisation
NASBA	-	Nucleic acid sequence based amplification

NG-MAST	-	*Neisseria gonorrhoeae* multi-antigen sequence typing
NGS	-	Next generation sequencing
NHANES	-	National Health and Nutrition Examination Surveys
OR	-	Odds ratio
ORF	-	Opening reading frame
PBP	-	Penicillin binding protein
PCR	-	Polymerase chain reaction
PEN	-	Penicillin
PFOR	-	Pyruvate-ferrodoxin oxidoreductase
PHC	-	Primary healthcare clinic
PID	-	Pelvic inflammatory disease
PMN	-	Polymorphonuclear leukocyte
PNA	-	Peptide nucleic acid
POC	-	Point of care
PPROM	-	Preterm premature rupture of membrane
RB	-	Reticulate body
RNA	-	Ribonucleic acid
rRNA	-	Ribosomal ribonucleic acid
ROS	-	Reactive oxygen species
SDA	-	Strand displacement amplification
SM	-	Syndromic management
SNP	-	Single nucleotide polymorphism
SP	-	Signal peptide
ST	-	Sequence type
STD	-	Sexually transmitted disease
STI	-	Sexually transmitted infection
TDM	-	Trehalose 69-dimycolate

TET	-	Tetracycline
TMA	-	Transcription-mediated amplification
TOCE	-	Tagging oligonucleotide cleavage and extension
TTSS	-	Type III secretion system
TV	-	*Trichomonas vaginalis*
TvENO	-	*Trichomonas vaginalis* α-enolase
TvGAPDH	-	*Trichomonas vaginalis* glyceraldehyde-3-phosphate dehydrogenase
TvLPG	-	*Trichomonas vaginalis* lipophosphoglycan
TVV	-	*Trichomonas vaginalis* virus
USA	-	United States of America
VDS	-	Vaginal discharge syndrome
VEC	-	Vaginal epithelial cells
VNTR	-	Variable-number tandem repeat
WHO	-	World Health Organization
XDR	-	Extremely drug resistance

LIST OF CONFERENCE CONTRIBUTIONS

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TRICHOMONAS VAGINALIS AND BACTERIAL CO-INFECTIONS IDENTIFIED IN REPRODUCTIVE AGE WOMEN

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SUMMARY

Sexually transmitted infections (STIs) continue to be a significant public health problem with an increased burden on women of reproductive age. These infections can be transmitted between humans by means of sexual activity including vaginal intercourse, oral sex and anal sex. Having a STI increases the risk of acquiring human immune-deficiency virus (HIV), hence the control of STIs is recommended for HIV prevention. The most common STI disease presentations to the public health setting in South Africa are male urethritis syndrome (MUS) and vaginal discharge syndrome (VDS). The main pathogens responsible for these two syndromes are: *Chlamydia trachomatis, Neisseria gonorrhoeae* and *Trichomonas vaginalis.* In the late 1990s the South African health department introduced the STI syndromic management approach into the primary health care setting. This approach aims to treat the common causes of STI syndromes through the use of specific treatment algorithms. It is cost effective because it allows treating the majority of STI patients without the need of laboratory diagnosis to determine the aetiological agent. People without any clear symptoms will remain untreated by the syndromic management approach. Little is known of the STI pathogens circulating in reproductive age women in the Tshwane region. The purpose of this study was to determine the prevalence of *T. vaginalis* and its co-infection in reproductive age women.

This study included self-collected vaginal swabs obtained from 117 consenting reproductive age women visiting either a public health clinic or a sexual private health clinic. The swabs

were cultured upon receipt in the laboratory on chocolate agar and in the InPouchTV for detection and diagnosis of *N. gonorrhoeae* and *T. vaginalis* respectively. The Nugent scoring system was used to diagnose bacterial vaginosis. The STI causing pathogens were detected on different molecular platforms which included Anyplex II STI-7 real-time PCR, GeneXpert CT/NG and GeneXpert TV.

The overall prevalence for both clinics of STIs was 13.7% (16/117) and for *T. vaginalis* specifically, a 10.3% (12/117) rate was observed. A co-infection rate of 2.6% (3/117) was observed in this study. *Trichomonas vaginalis* occurred mostly with *C. trachomatis* (12.5%) followed by *N. gonorrhoeae* (6.3%). Most co-infections were observed in women of younger than 30 years old. Age was not significantly associated with the prevalent *T. vaginalis* infection in this study but being unmarried showed a significant association (p-value=0.038) with the prevalent infection in both clinics. The high rates of *Trichomonas vaginalis* and coinfections with *C. trachomatis* and *N. gonorrhoeae* observed in the asymptomatic women visiting the two clinics provide evidence that in certain key groups the simultaneous screening for all three pathogens should be performed.

CHAPTER 1

INTRODUCTION

1.1 Introduction

A sexually transmitted disease (STD) or sexually transmitted infection (STI) is an illness that has the likelihood of transmission between humans by means of human sexual activity, including vaginal intercourse, oral sex and anal sex [Centers for Disease Control and Prevention (CDC), 2013]. Sexually transmitted infections are a major health problem globally (Mckechine *et al.,* 2009). Worldwide, an estimated 340 million cases of curable STIs, which include chlamydial infection, gonorrhoeae, trichomoniasis and syphilis occur annually with an incidence that is increasing in many parts of the world (Mckechine *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 of any age 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 CDC, an estimated 19 million new cases occur each year in the United States of America (USA) and almost half of them are in the 15 to 24 years age group (Forhan *et al.,* 2009).

Most of the STIs are asymptomatic but the consequences are serious (Samraa et al., 2011) as 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 (Peeling *et al.,* 2006). Women who are infertile are viewed in some societies as worthless (Peeling *et al.,* 2006). Sexually transmitted infections can have serious adverse outcomes in pregnancy for both the woman and the foetus or infant (Forhan *et al.,* 2009).

Prevention of sexually transmitted infections in this reproductive 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 depending on geographical, social and

economic situations (Joyee *et al.,* 2005). Progress in vaccine development for most STIs is still quite limited (Nusbaun *et al.,* 2004). In the meantime pathogens, such as *Chlamydia trachomatis, Neisseria gonorrhoeae* and *Trichomonas vaginalis* have been linked to an increased transmission of HIV and also have the potential to cause a significant morbidity on their own (Fleming & Wassarheit, 1999; Joyee *et al.,* 2005).

South Africa has a high burden of STIs and manages these infections using a syndromic approach (Johnson *et al.,* 2005*).* A study conducted in South Africa investigated trends in *T. vaginalis* prevalence among male urethral discharge syndrome/vaginal discharge syndrome (MUDS/VDS) patients in an annual STI aetiological survey at a public sector primary healthcare clinic (PHC) in Johannesburg from 2007 to 2012 (Lewis *et al.,* 2013). A total of 1 218 men with MUDS and 1 232 women with VDS were enrolled into the six annual aetiological surveys. *Trichomnas vaginalis* infection was detected in 6.1% (74/1 232) of men and in 23.6% (291/1 232) of women (Lewis *et al.,* 2013).

Trichomonas vaginalis is an obligate flagellated parasite, which cannot live without close association with the vaginal, urethral or prostatic tissues (Arora, 2005). Humans are the only known host with the trophozoite transmitted principally *via* vaginal sexual intercourse and rarely *via* fomites (Wilkerson, 2011). Asymptomatic *T. vaginalis* infection was detected in 47% of 780 women attending an obstetrics and gynaecology clinic in India (Yadav *et al.,* 2005) and in 58% of 189 women attending a family planning clinic in rural South Africa (Wilkinson *et al.,* 1997).

A study by Sutton (2007) in United States identified the following as risk factors for women to acquire a STI: increasing age, African-American race, multiple sexual partners, sex with casual or older partners, lower educational level, poverty, injection drug use and crack cocaine use. The public health importance of trichomoniasis is highlighted by the fact that the infection in women is associated with adverse outcomes, which include pelvic inflammatory disease, concurrent vaginitis and other STIs, pregnancy complications, infertility, cervical cancer and the enhanced risk of HIV-1 acquisition and transmission (Weinstock *et al.,* 2004; McClelland, 2008; Fichorova, 2009).

In women, trichomoniasis encompasses a broad range of symptoms ranging from severe inflammation and irritation with frothy foul-smelling discharge to a relatively asymptomatic

carrier state (Imam *et al.,* 2007). A study in men utilising transcription-mediated amplification (TMA) demonstrated significant co-detection of *N. gonorrhoeae* with *T. vaginalis* and a further association with *C. trachomatis* (Huppert *et al.,* 2007). The main clinical manifestation of trichomoniasis is vaginitis, urethritis and prostatitis (Imam *et al.,* 2007). Pregnant women infected with *T. vaginalis* may be at the risk of adverse birth outcomes, such as premature rupture of membranes, premature labour, low birth weight, post-abortion or post-hysterectomy infection and have enhanced chance to present with neoplastic changes in the cervical tissues (Uneke *et al*., 2006). Treatment options for trichomoniasis infection are limited and include two oral 5- nitromidazole drugs namely metronidazole and tinidazole (Schwebke and Barrientes, 2006). Tinidazole has a biological half-life twice as long as that of metronidazole, better *in vitro* activity against trichomonads and is well tolerated (Crowell *et al.,* 2003).

As earlier stated, co-infection of *Trichomonas vaginalis* with other STIs is common; associations between *T. vaginalis* infection and chlamydia, gonorrhoea, syphilis and herpes simplex virus-type 2 (HSV-2) have been described and the correlation between an increased risk of HIV transmission and *T. vaginalis* infection has been well supported by the literature (Miller *et al.,* 2005). Human immuno-defiency virus appears to be transmitted more easily with *T. vaginalis* infection (Schwebke, 2005). It has been shown that treatment of *T. vaginalis* reduces viral shedding and potentially HIV transmission (Kissinger *et al.,* 2009). Despite the risks associated with *T. vaginalis* infection trichomoniasis was not a reportable disease when the National Health and Nutrition Examination Surveys (NHANES) surveys were conducted from 2001 to 2004 in United States and often was not included in routine testing (Van der Pol, 2007).

Another possible co-infection with *T. vaginalis* is bacterial vaginosis which have been estimated to affect as many as one-quarter to one-third of sexually active females worldwide and are often found concurrently (Atashili *et al.,* 2008). *Trichomonas vaginalis* colonisation is increased in the presence of bacterial vaginosis-defining phenomena, such as elevated amine production, loss of facultative lactobacilli and increased pH (Brotman *et al.,* 2011).

The World Health Organization (WHO) recommends that STIs be managed at the first point of contact with the health services using the syndrome approach (Alder, 1996). 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 (Alder, 1996; Pepin *et al.,* 2004; van der Eem *et al.,* 2016). Syndromic management can result in as much as 98% overtreatment for *Chlamydia* and gonorrhoeae 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 has led to a need for rapid and reliable assays capable of simultaneously identifying multiple pathogens in a single clinical sample (Khan *et al.,* 2005; Lee *et al.,* 2007).

In South Africa the STI syndromic management approach was introduced to primary health care approximately 18 years ago (Lewis & Maruma, 2009). Flowcharts are produced, which guide healthcare workers in the correct implementation of the syndromic management of STIs (Lewis & Maruma, 2009). The established sexually transmitted pathogens implicated in the aetiology of urethritis and vaginal discharge includes *N. 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).

There is a need for periodic surveillance to assess the microbiological causes of the various STI syndromes; the prevalence of the agents, the antimicrobial susceptibility patterns for key bacterial STI pathogens and the epidemiology. The results from this study will assist in the understanding of the prevalence of the common aetiological pathogens and its co-infections in the Pretoria region.

The objectives of this study were:

1 To collect vaginal swabs from reproductive age women who attend routine check up

2 To diagnose bacterial vaginosis using the Nugent scoring system

3 To diagnose trichomoniasis using microscopy, culture and a molecular method

4 To detect and identify *Neisseria gonorrhoea* using culture and biochemical tests

5 To determine the genetic relatedness of *N. gonorrhoeae* using NG-MAST and to determine antimicrobial susceptibility profiles of the *N. gonorrhoeae*

6 To molecularly detect *T. vaginalis* and bacterial co-infections using real time PCR assays

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Sexually transmitted infections (STIs) are extraordinarily common place, with an estimated 340 million new cases of "curable" infections occurring each year worldwide in men and women aged between 15 to 49 years (WHO, 2013). These infections include those caused by bacterial and protozoal agents that have been treated by appropriate antibiotics and chemotherapeutic agents for more than 40 years, namely syphilis, gonorrhoea, chlamydia and trichomoniasis (Gross & Tyring, 2011). The largest proportion of STIs occur in developing nations, led by South and Southeast Asia, followed by sub-Saharan Africa, Latin America and the Caribbean (WHO, 2001). Sexually transmitted infections impose a major health burden, particularly in developing countries, such as South Africa, where the prevalence of human immunodeficiency virus type 1 (HIV-1) infection is high (Johnson & Criss, 2011). In South Africa, most new HIV infections are sexually transmitted and women are at higher risk of infection than men (Wand & Ramjee, 2012). As STIs are associated with increased susceptibility to HIV infection, they have likely played a central role in facilitating the spread of HIV (Wand and Ramjee, 2012).

Trichomonas vaginalis is the most common non-viral STI in the world (Johnston & Mabey, 2008). *Trichomonas vaginalis* infection was more prevalent (>11%) than *Chlamydia trachomatis* (9.2%) and *Neisseria gonorrhoeae* (2.2%) infections combined among women in the United States from July to November 2010 (Ginocchio *et al.,* 2012). In the United State of America (USA), it is estimated that 3.1% of women of reproductive age are infected with *T. vaginalis* and prevalence rates up to 50% have been reported in certain populations (Meites *et al.,* 2013). *Chlamydia trachomatis* infection is one of the most common notifiable diseases in the United States with >1.3 million infections reported to the Centers for Disease Control and Prevention (CDC) in 2010 (Adekoya, 2010). Gonorrhoea typically presents as urethritis in men and cervicitis in women but when left untreated can result in severe sequelae, such as infertility (Ndowa & Lusti-Narasimham, 2012).

Neisseria gonorrhoeae infections were the second most common STI in the United States (CDC, 2013). Gonorrhoea infections continue to be a public health problem worldwide and control efforts have been complicated because of the ability of the bacterium to develop resistance to all first-line antimicrobial drugs used in treatment, including penicillins, tetracyclines and fluoroquinolones (Duylinh *et al.,* 2012). Therefore, gonorrhoea may become untreatable in certain circumstances as the organism has emerged as a "superbug" (Tapsall *et al.,* 2010).

Mycoplasma hominis and *Ureaplasma* spp., including *U. parvum* and *U. urealyticum*, are collectively known as genital mycoplasmas and are found in the vaginal milieu of up to 80% of pregnant and non-pregnant women (Bayraktar *et al.,* 2010). Ureaplasmas are reported to be more prevalent than other mycoplasmas in the female urogenital tract, with *U. parvum* found more often than *U. urealyticum* (Patel & Nyirjesy, 2010). *Ureaplasma parvum* might be present in bacterial loads leading to adverse pregnancy outcomes and produce asymptomatic infections of the upper genital tract in women as frequently as *U. urealyticum* (Kasprzykowska *et al.,* 2013). The greater virulence reported for *U. urealyticum* in some conditions might be attributed to its superior capability of acquiring genes horizontally from various ureaplasma strains (Paralanov *et al.,* 2012).

Bacterial vaginosis (BV) is the most common cause of unpleasant vaginal odour and discharge in women of reproductive age (Brotman, 2011). The essential participants in pathological polymicrobial associations, which could be used as markers for BV, are *Gardnerella vaginalis* and the anaerobic bacterium, *Atopobium vaginae* (Turovskiy *et al.,* 2011). It is not clear yet if BV is a sexually transmitted disease, but it is more common in promiscuous women with multiple and/or new sexual partners or with female partners and sex during menses (Marrazzo *et al.,* 2010). Bacterial vaginosis can be an independent risk factor for the acquisition of any other sexually transmitted infection (Gallo *et al.,* 2012).

Sexually transmitted infections continue to be a significant public health problem with an increased burden on women of reproductive age (Rours *et al.,* 2010). Sexually transmitted infections have been associated with a wide spectrum of complications, such as urethritis and epididymitis in men and cervicitis in women (Walker *et al.,* 2012). According to the recent South African sentinel surveillance data, the prevalence of some STIs, such as *T. vaginalis, C. trachomatis* and *N. gonorrhoeae* has not declined over the past 11 years (Mahmood and

Sanioto, 2011). This current study was focused on *Trichomonas vaginalis* and its co-infections. *Trichomonas vaginalis* was thought to be a silent asymptomatic pathogen until studies proved it to be associated with adverse complications, such as preterm birth, pelvic inflammatory disease and infertility in reproductive age women (Cherpes *et al.,* 2006). *Neisseria gonorrhoeae* has been proven to be the second most common sexual transmitted infection; both *T. vaginalis* and *N. gonorrhoeae* associated complications can affect the female and also an unborn baby (Cherpes *et al.,* 2006). Due to differences in clinical presentation, it has been difficult to diagnose, so this study will employ the most recent available methods to detect *T. vaginalis* and *N. gonorrhoeae* molecularly.

2.2 *Trichomonas vaginalis*: General characteristics of the organism

Among sexually transmitted infections, *T. vaginalis* is the most common non-viral STI worldwide that causes trichomoniasis (WHO, 2011). Trichomonads are anaerobic flagellated protozoa inhabiting the urogenital and digestive tract of animals and humans (Honigberg & Brugerolle, 1990). Trichomoniasis, a STI caused by the protozoa *Trichomonas vaginalis*, is the most common curable STI in young, sexually active women (Weinstock *et al.,* 2004). It can cause symptoms ranging from mild irritation to severe inflammation; however, approximately 50% of infected people remain asymptomatic (Schirm *et al.,* 2007). Trichomoniasis has often been considered a relatively benign infection, but given its association with other STIs, particularly HIV and preterm delivery it is an important public health problem (Schwebke & Burgess, 2004).

Trichomonas vaginalis is a flagellated protozoan, possessing five flagella, four of which are located at its anterior portion; the fifth flagellum is incorporated within the undulating membrane of the parasite which is supported by a slender non-contractile structure (Eshet *et al.,* 2013). This parasite varies in size and shape, with the average length and width being 13 μm and 10 μm (Eshet *et al.,* 2013). *Trichomonas vaginalis* is characterised by the presence of a parabasal body: Golgi associated with a kinetosome; axostyle and an undulating membrane (Schwebke & Burgess, 2004). Humans are the only known host with the trophozoite transmitted *via* sexual intercourse and fomites; both males and females are infected but the majority of cases were reported among females who also more frequently present with symptomatic infection compared to males (Jamali *et al.,* 2006). Pregnant women infected with the *T. vaginalis* parasite may be at risk of an adverse birth outcome, like post-abortion or post-

hysterectomy infection, as well as infertility and having enhanced predisposition to neoplastic transformation of cervical tissues (Chinyere *et al.,* 2010).

2.2.1 Classification of *T. vaginalis*

Trichonomas 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). The *Diplomonads* also belong to the *Excavata* and is most closely related to the *Parabasalids* (Gerbod *et al.,* 2004; Schwebke & Burgess, 2004) (Table 2.1). *Giardia lamblia* is a well-known species of the *Diplomonads* (Gerbod *et al.,* 2004). It is commonly known as "Beaver Fever", which causes severe diarrhoea (Gerbod *et al.,* 2004; Schwebke & Burgess, 2004). The members of the order *Trichomonadida* has four to six flagella, which are free or attached to an undulating membrane (Schwebke & Burgess, 2004).

Table 2.1: Scientific classification of *Trichomonas vaginalis* (Schwebke and Burgess, 2004)

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

2.2.2 Physiology and metabolism of *T. vaginalis*

Trichomonads grow best at 35°C to 37°C in anaerobic conditions (Adl *et al.,* 2012). Among the parasitic trichomonads, several species inhabit the oral, digestive and urogenital tracts of invertebrate and vertebrate hosts including humans, livestock and pets (Julia *et al.,* 2014).

Minimally elevated levels of oxygen can; however, improve growth as long as the partial pressure of carbon dioxide remains high (Huang *et al.,* 2014). *Trichomonas vaginalis* import pyruvate and malate to generate adenosine triphosphate (ATP) through substrate-level

phosphorylation without the use of oxygen as the terminal electron acceptor, releasing hydrogen and acetate as end products (Ginger *et al.,* 2010; Műller *et al.,* 2012).

Trichomonas vaginalis has multiple proteinases, mainly of the cysteine type (Cárdenas-Guerra *et al.,* 2013). In the *T. vaginalis* genome sequence, 48 genes encoding an enzyme papain-like cysteine type were found (Carlton *et al.,* 2007). Up to 23 spots with proteolytic activity between 23 and 110 kDa were detected using two-dimensional (2-D) substrate gel electrophoresis (Neale & Alderete, 1990). However, most of these spots were encoded by only nine distinct cysteine type genes (Ramo'n Luing *et al.,* 2010). Erythrocytes are an essential source of nutrients, such as lipids and iron for trichomonads (Rendòn-Maldonado *et al.,* 1998). These parasites phagocytise and lyse erythrocytes by a contact-dependent mechanism (Rendòn-Maldonado *et al.,* 1998). Amongst the molecules possibly involved in *T. vaginalis* haemolysis are pore forming proteins and phospholipase-A-like proteins identified as cytolytic factor (Lubick & Burgess, 2004).

2.2.3 Pathogenesis and virulence factors of *T. vaginalis*

Trichomonas vaginalis responds to drastic environmental changes (e.g., temperature, microflora, pH, iron, polyamines, zinc, host immune responses, and other unknown factors), modulating the expression of multiple genes, including those encoding virulence factors, to maintain a chronic infection (Lehker and Alderete, 2000). Genes encoding for superoxide dismutases, thioredoxin reductases, peroxiredoxins and rubrerythrins have been identified in the trichomonad genome (Carlton *et al.,* 2007). To establish an infection in humans, *T. vaginalis* binds and degrades the vaginal mucous and specifically adheres to vaginal and cervical epithelial cells (cytoadherence) (Lehker & Alderete, 2000). However, *T. vaginalis* does not undergo the same morphological changes as with other cell types as they do with vaginal epithelial cells (VECs) (Vazquez-Carrillo *et al.,* 2009). Upon contact with VECs, *T. vaginalis* undergoes a drastic morphological shift, changing from the usual pear shape to an amoeboid form with an increase in the expression of adhesins and a release of chemo-attractant molecules which attracts more parasites to the site of infection (Vazquez-Carrillo *et al.,* 2011). The morphological transformation leads to the formation of areas where the parasite and the target cell become tightly associated (Kucknoor *et al.,* 2005). Parasite morphology is directly related to cytoskeletal structure and changes in parasite shape are mediated by the redistribution of its molecular components (Addis *et al.,* 1998). As revealed

by scanning electron microscopy, the morphology of *T. vaginalis* in contact with DU145 prostatic cells retains a pear-like shape (Kucknoor *et al.,* 2005) (Fig. 2.1).

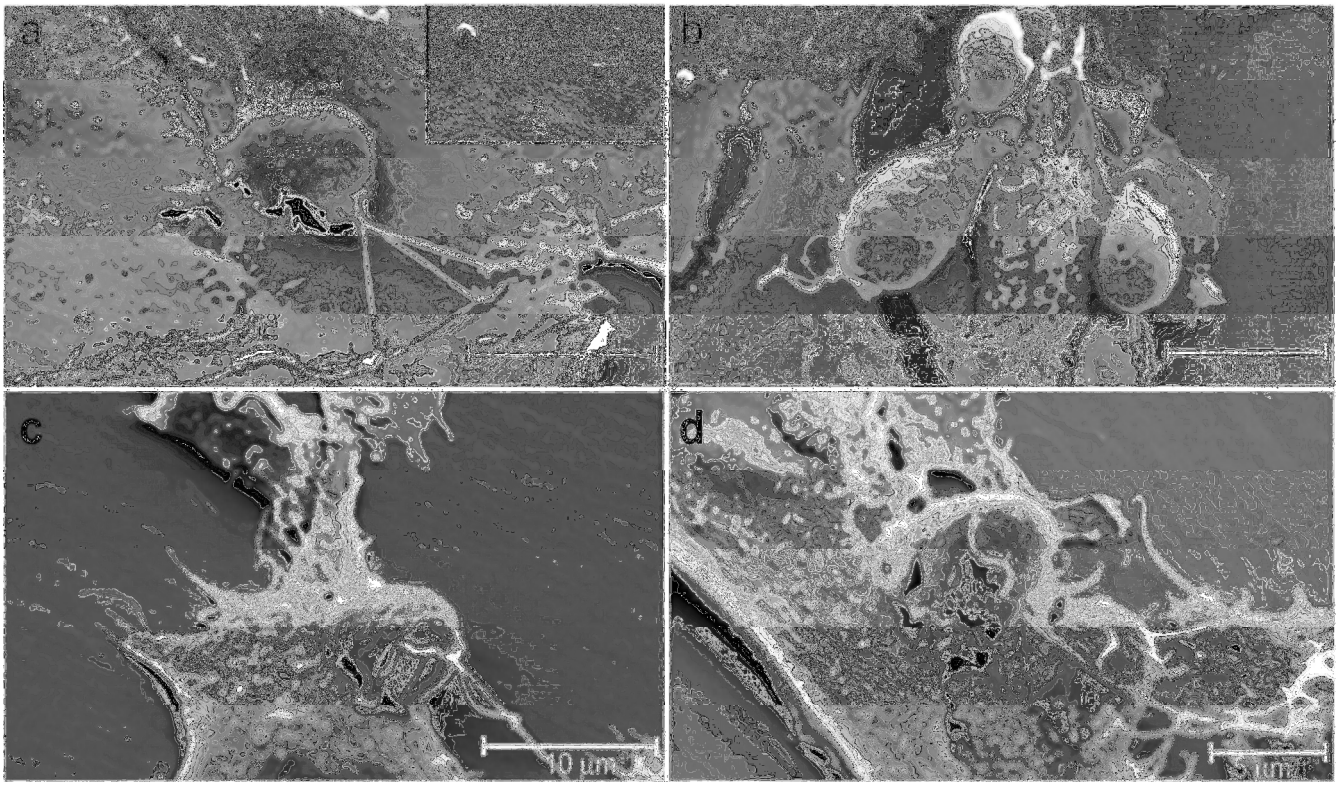

Figure 2.1: Morphological appearance of *T. vaginalis* in contact with DU145 prostatic cells at different time points. A. Trophozoites were incubated with DU145 cells for a) 5 min, b) 30 min, c) 60 min, and d) 90 min. DU145 prostatic cells before interacting with *T. vaginalis* were used as a control (inset of panel a). The majority of parasites attached to DU145 cells retain a pear-like shape with four flagella, an undulating membrane, and the axostyle. Some parasites showed a few pseudopods (panel b and c). After 5 min of interaction, the lytic activity of the parasites is visible as seen by the disruption of the cell monolayer (Kucknoor *et al.,* 2005).

Trichomonas vaginalis possesses an actin cytoskeleton and several of its protein components have already been characterized including actin, an actin-binding protein that actively participates in the morphological transformation (Vazquez-Carrillo *et al.,* 2011). An actin-binding protein is involved in mediating the redistribution of actin and two fibrins, which belongs to the actin-bundling proteins and expressed in the presence of zinc (Vazquez-

Carrillo *et al.,* 2011). *Trichomonas vaginalis* actin is evenly distributed throughout the cytoplasm when the cell is pear-shaped (Figueroa-Angulo *et al.,* 2012).

Parasite cytoadherence is a prerequisite for establishing and maintaining a chronic infection in the human urogenital tract (Lehker & Alderete, 2000). Cytoadherence is a complex process that involves not only surface proteins "adhesins" and glycoconjugates but also proteins in the cytoskeleton (consisting of microtubules and microfilaments), the receptors for extracellular matrix (ECM) proteins (laminin, fibronectin, and collagen), and signal transduction and autophagy processes (Moreno-Brito *et al.,* 2005). Five *T. vaginalis* surface proteins that interact with the host cell surface have been characterised as adhesins (AP120, AP65, AP51, AP33, and AP23) (Moreno-Brito *et al.,* 2005). Four of the surface proteins are metabolic enzymes [pyruvate ferredoxin oxidoreductase (PFOR), malic enzyme and the A and B succinyl-CoA synthetase subunits], suspected of having dual localisation inside trichomonads (hydrogenosomes and parasite surface) (Moreno-Brito *et al.,* 2005). These adhesins have been identified and characterised as multifunctional proteins that show different functions depending on their localisation regulated by iron, acting as metabolic enzymes in hydrogenosomes and the cytoplasm or as adhesins on the parasite surface (Moreno-Brito *et al.,* 2005). These hydrogenosomal enzymes lack enzymatic activity when localised at the parasite surface, performing additional tasks as virulence factors in adherence (Moreno-Brito *et al.,* 2005).

Cysteine proteinases (CPs) localized to the parasite surface, such as TvCP30 and TvCP62, the surface lipophosphoglycan (TvLPG) and several ECM receptors, are also involved in trichomonal cytoadherence (Hernandez *et al.,* 2004). The TvCP30 participates in cytoadherence, is immunogenic and is present in vaginal secretions in patients with trichomoniasis and is active at the pH (>5) and temperatures (>37°C) found in the vagina during infection (Bastida-Corcuera *et al.,* 2005). The CPs degrades proteins of the vaginal milieu such as collagen (Coll) IV, fibronectin (Fn) and haemoglobin (Hb) (Mendoza-Lopez *et al.,* 2000). Other metabolic proteins with alternative non-enzymatic function are glyceraldehyde-3-phosphate dehydrogenase (TvGAPDH) and α-enolase (TvENO-1) (Mendoza-Lopez *et al.,* 2000). These proteins are also found on the *T. vaginalis* surface, but their sequences lack trehalose 69-dimycolate (TMD) and signal peptides (SP) (Lama *et al.,* 2008). Glyceraldehyde-3-phosphate dehydrogenase and α-enolase exhibit ligand binding activity, a non-enzymatic function that may play an important role in colonisation and

invasion, probably under distinct host environments (Alderete and Arroyo, 2008). Surface localised TvEN +O-1 binds plasminogen, and its synthesis is increased following trichomonads cellular contact with VECs (Mundodi *et al.,* 2008).

Light and electron microscopy studies have helped to demonstrate that *T. vaginalis* is a phagocytic cell that is able to efficiently ingest and degrade Döderlein's lactobacilli, vaginal and cervical epithelial cells, leukocytes, erythrocytes, yeast, spermatozoids and prostatic cells (Vazquez-Carrillo *et al.,* 2009). Although the mechanism of phagocytosis has not been fully elucidated, at least two different pathways have been observed: (i) the pathway used by phagocytes in which pseudopodia are extended toward the target cell and (ii) a sinking process without membrane extension (Pereira-Neves & Benchiomol, 2007). Two surface proteins have been described as putative adhesins for erythrocytes, whereas a mannose receptor on the parasite surface has been described for yeast phagocytosis (Midlej & Benchiomol, 2010). Changes in the distribution of the actin cytoskeleton have also been reported, which may facilitate the ameboid morphological transformation observed during phagocytosis (Pereira-Neves & Benchiomol, 2007). Thus, it has been suggested that phagocytosis may be considered to be a virulence mechanism in addition to a mechanism for the acquisition of iron, lipids, nucleotides, and other nutrients (Fiqueroa-Angulo *et al.,* 2012).

2.2.4 *Trichomonas vaginalis* in pregnant and non-pregnant women

Trichomonas vaginalis infections have been associated with poor reproductive outcomes, such as premature birth and low birth weight (Schwebke & Burgess, 2004). This parasite is considered to be sexually transmittable and sometimes related to low socio-economic levels (Rughooputh & Greenwell, 2005). Trichomoniasis is associated with adverse reproductive sequelae including pelvic inflammatory disease in women and infertility in women and men (Fichorova, 2009). Risk factors for infection among women include increasing age, greater number of life time sexual partners, sex with casual or older partners, lower educational level, poverty, injection drug use and crack cocaine use (Sutton *et al.,* 2007). *Trichomonas vaginalis* is recognised as a risk factor for the acquisition and transmission of HIV (Anderson *et al.,* 2012).

Trichomoniasis is the most common curable STI in young, sexually active women (Weinstock *et al.,* 2004). Among HIV-infected women, *T. vaginalis* prevalence is estimated

to be between 5% and 30% depending upon geographical location as HIV prevalence differs in different geographical locations and races for example, African-American men and women have a high rate of HIV infection (Seth *et al.,* 2008). Interestingly *T. vaginalis* infections differ from other STIs in that the prevalence increases with increasing age among women under the age of 50 years old (Miller *et al.,* 2005). In women above 50 years, most women are no longer as sexually active (Miller *et al.,* 2005) or are more settled in established relationships, hence leading to a low prevalence.

2.2.5 Antimicrobial resistance of *T. vaginalis*

In practice, treatment for *T. vaginalis* is exclusively based on 5-nitroimidazoles, like metronidazole or tinidazole (Schwebke & Barrientes, 2006). Although metronidazole was introduced for the treatment of *T. vaginalis* infection more than 50 years ago, it still is a highly reliable antimicrobial and antimicrobial resistance is still relatively rare (Schwebke & Barrientes, 2006). *In vitro* antimicrobial resistance has been documented in 4% of vaginal culture specimens collected from STI clinics and *in vivo* antimicrobial resistance has remained infrequent to date (CDC, 2013). Antimicrobial resistance can be overcome by higher doses of metronidazole or by prescribing tinidazoles instead but not always (Goldman *et al.,* 2009). Metronidazole resistance in *T. vaginalis* constitutes a serious problem because alternative treatments regiments, such as a single higher dose of metronidazole are rather ineffective (Goldman *et al.,* 2009).

The antiparasitic effect of metronidazole is based upon its activation in the *T. vaginalis* mitochondrion-relict organelle, the hydrogenosome (Conrad *et al.,* 2013). This activation generates nitro-radicals that are locally toxic to the trichomonas cells by interfering with proteins and protein trafficking (Conrad *et al.,* 2013). Laboratory-generated resistance is associated with the down-regulation of enzymes thought to reduce metronidazole, such as pyruvate-ferrodoxin oxidoreductase (PFOR) and ferredoxin, as well as shrinking of the hydrogenosomes, a mitochondrion-related organelle where these enzymes are located in *T. vaginalis* (Wright *et al.,* 2010). However, resistant clinical isolates harbour normal-sized hydrogenosomes and do not exhibit reduced transcription of the PFOR or ferredoxin gene (Wright *et al.,* 2010).

2.3 *Neisseria gonorrhoeae*: Classification of the organism

Neisseria gonorrhoeae are prokaryotes and are grouped in the domain *Bacteria* (Euzéby, 2012). 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, habitats and morphologies (Euzéby, 2012).

The *Neisseriales* contains a group of Gram-negative, non-spore forming, aerobic and mesophilic bacteria (Adeolu & Gupta, 2013). The Genus *Neisseria* includes at least 25 species (Table 2.2) based on 16S rRNA gene sequence information (Bennett *et al.,* 2012).

Table 2.2: Scientific classification of *Neisseria gonorrhoeae* (Bennett *et al.,* 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. meningitides, 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 surface 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 disease presentations (Adeolu & Guptas, 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 & Gupta, 2013

2.3.1 General characteristics of *N. gonorrhoeae*

Neisseria gonorrhoeae is the aetiological agent of the second most common STI globally after *T. vaginalis* (WHO, 2012). The number of gonococcal infections is rapidly increasing, especially because individuals are often asymptomatic (WHO, 2012). This condition

contributes to the spread of the pathogen, but also to the silent progression of the infection to more serious clinical conditions, such as pelvic inflammatory disease (Ison & Alexander, 2011).

Neisseria gonorrhoeae is an aerobic, Gram-negative, non-motile and non-spore-forming coccus (Adeolu & 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, 2005). These bacteria are structurally differentiated from other cocci by their 'kidney-shaped' diplococcic forms (Tønjum, 2005).

Neisseria gonorrhoeae is a fastidious bacterium that exhibit complex growth requirements when cultured *in vitro* (WHO, 2013). The following enriched media are used for the growth of *N. gonorrhoeae*: Thayer-Martin, modified Thayer-Martin, Lewis-Martin and New York city agar (WHO, 2013). These media have a chocolate agar base for growing fastidious organisms and contain antibiotics, such as vancomycin, colistin, amphotericin B and trimethoprim that inhibit the growth of yeasts and other bacteria (Spence *et al.,* 2008). Chocolate agar medium is typically augmented with Kellogg's supplement or isoVitalex (Becton-Dickinson, Franklin Lakes, NJ) to enhance the growth of gonococci on plates (Spence *et al.,* 2008). *Neisseria gonorrhoeae* forms opaque colonies *in vitro* and has the ability to form biofilms on glass surfaces as well as on primarily and immortalised urethral and cervical epithelial cells (Youssef *et al.,* 2009; Phillips *et al.,* 2012).

Neisseria gonorrhoeae is cultured at 37^{o}C and does not survive at temperature below 25^{o}C (Spence *et al.,* 2008). Colonies appear on plates within 24 h to 48 h of incubation but viability is rapidly lost due to autolysis after depletion of glucose from the medium (WHO, 2013). After further incubation colonies may reach 3 mm in diameter and become less smooth and are pinkish-brown in colour (WHO, 2013).

Neisserial lipopolysaccharides (LPS) are unique in that it has a highly-branched basal oligosaccharide structure and the absence of repeating O-antigen subunits (Edwards & Butler, 2011). During growth, the bacterium releases outer membrane fragments called "blebs" which contains biologically active proteins (WHO, 2013). The bacterium has fimbriae, which is a proteinaceous appendage that is thinner than a flagellum (WHO, 2013). There are four types of *N. gonorrhoeae* based on the presence of fimbriae called: T1, T2, T3 and T4 (Fox *et al.,*

2014). *Neisseria gonorrhoeae* can utilise glucose, lactose or pyruvate as sole carbon source, but cannot metabolise other carbohydrates (Phillips *et al.,* 2012). *Neisseria*'s cell wall consists of an outer membrane made up of outer membrane proteins, lipids, lipopolysaccharides, an inner peptidoglycan matrix and a cell membrane (Edwards & Butler, 2011). Porin proteins cover the outer membrane and forms aqueous channels that allow the exchange of antibiotics, dyes, ions and other larger compounds to occur (Cooper *et al.,* 2012).

The *Neisseria gonorrhoeae* genome contains homologues of enzymes involved in peptidoglycan recycling and the levels of turnover are consistent with a certain level of recycling occurring in gonococci (Garcia & Dillard, 2008). Gonococcus genomes contain approximately 11 opacity (*opa)* loci, encoding seven to nine unique Opa proteins (de Jonge *et al.,* 2003). In general *N. gonorrhoeae* has 2 662 coding sequences and a variable plasmid complement (Helena & Thompson, 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).

2.3.2 Pathogenesis and virulence factors

The presence of gonococcus at human mucosal surfaces initiates the recruitment of abundant polymorphonuclear leukocytes (neutrophils; PMNs) to the site of infection (Criss & Seifert, 2008). Interactions between the gonococcus and human cells are modulated by a variety of bacterial surface structures, including type IV pili (fimbriae), lipooligosaccharide (LOS), porins, and opacity-associated (Opa) proteins (Fox *et al.,* 2014). The opa proteins are 25 kDa to 30 kDa outer membrane proteins that were named for their ability to confer an opaque morphology on gonococcus colonies (Swanson, 1978). The Opa proteins share a conserved β-barrel structure but differ in the sequence of extracellular loops, which dictate Opa receptor specificity (Fox *et al.,* 2014). Most Opa proteins interact with one or more human carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) (OpaCEA) (Sadarangani *et al*., 2011). A subset of Opa proteins binds to heparin sulfate proteoglycans on epithelial cells or indirectly engages integrins by binding vitronectin (Fox *et al.,* 2014). The Opa proteins can also recognise lacto-*N*-neotetraose moieties on LOS (Levan *et al.,* 2012). The Opa expression is phase variable due to slipped-strand mispairing at pentameric repeats in the 5 end of the *opa* genes (Levan *et al.,* 2012). Thus, gonococci can express anywhere

from zero to theoretically all Opa proteins (Fox *et al.,* 2014). The Opa variation occurs at a rate of 10^{-3} to 10^{-4} variants/cell/generation *in vitro* and changes in Opa expression occur during experimental human male urethral infection and in the female mouse genital tract (Simms & Jerse, 2006).

Expression of the Opa proteins, particularly opacityCEA (OpaCEA), affects polymorphonuclear leukocytes (PMN) antibacterial activities and gonococci survival after exposure to PMNs (Criss & Seifert, 2008). Activated PMNs assemble the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme to generate reactive oxygen species (ROS), e.g., the oxidative burst (Roos *et al.,* 2003). Most Opa-positive (Opa^+) gonococci stimulate primary human PMNs and promyelocytic HL-60 cells to produce ROS (Teufelhofer *et al.,* 2003). In contrast, predominantly Opa-negative (Opa^-) gonococci not only fail to induce PMN ROS production but also suppress the ability of PMNs to produce ROS after exposure to other stimuli such as active NADPH oxidase (Criss & Seifert, 2008). The Opa proteins also affect gonococcus phagocytosis and killing by PMNs (Teufelhofer *et al.,* 2003). Human PMNs in suspension can bind and phagocytose unopsonized gonococci only if the bacteria express OpaCEA proteins (Teufelhofer *et al.,* 2003). Gonococcus phagocytosis by adherent PMNs is more efficient when the bacteria are Opa^+, but Opa^- gonococci are also internalized (Criss *et al.,* 2009). Regardless of the system used, Opa^+ gonococci are more sensitive than Opa^- gonococci to killing by human PMNs (Sarantis & Gray-Owen, 2007).

2.3.3 Immune response to *N. gonorrhoeae* infections

The clinical symptoms of gonococcal infections are caused primarily by the inflammatory infiltrate of neutrophils responding to cytokines expressed by induction of the innate immune system (Cooper *et al.,* 2012). *Neisseria gonorrhoeae* typically triggers an intense inflammatory response characterised by an influx of neutrophils into the genital tract, yet natural gonococcal infection does not induce a state of specific protective immunity (Liu & Russel, 2011). Hence recovery from each incident of infection does not leave an effective protective immunity against the organism (Liu & Russel, 2011). Lack of an adaptive immune response to *N. gonorrhoeae* probably contributes to the continuing prevalence of the sexually transmitted infection and challenges the development of a vaccine against it (Zhu *et al.,* 2011).

Neisseria gonorrhoeae infection triggers a potent local inflammatory response driven by PMNs (Hedges *et al.,* 1998; Johnson & Criss, 2011). Polymorphonuclear leukocytes are phagocytic cells that are an important component of the host innate immune response and are the first responders to bacterial and fungal infections (Johnson & Criss, 2011). Polymorphonuclear leukocytes 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 & 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 & Song, 2010).

2.3.4 Antimicrobial susceptibility testing for *N. gonorrhoeae*

Antimicrobial resistance (AMR) is making the clinical management of infections, such as gonorrhoea increasingly difficult (Davies *et al.,* 2013). In between the time of the discovery of penicillin and the emergence of multidrug resistant *N. gonorrhoeae* (MDR-NG) and extensively drug resistant *N. gonorrheae* (XDR-NG) strains, gonorrhoea was considered as an unpleasant infection, but not perceived as a serious infection as it was easy to treat (Ohnishi *et al.,* 2011). Experts increasingly describe *N. gonorrhoeae* as becoming an untreatable superbug (Unemo & Nicholas, 2012; WHO, 2012) because of reports of MDR-NG and XDR-NG strains resulting in treatment failures with extended spectrum cephalosporins (ESCs), such as cefixime and ceftriaxone reported from Europe, North America, Asia and Africa (Lewis *et al.,* 2013). If these infections become untreatable then complications, including pelvic inflammatory disease, ectopic pregnancy, tubal infertility, neonatal eye infections and consequences such as facilitation of HIV co-transmission, will become more common (Allen *et al.,* 2011).

The quinolones are broad-spectrum antibacterial agents that are receiving attention as resistance develops to other compounds (Drlica *et al.,* 2009). These compounds have two essential bacterial enzymes as their targets: DNA gyrase (topoisomerase II) and DNA topoisomerase IV (Drlica *et al.,* 2009). In brief, resistance to ciprofloxacin (CIP) is usually due to amino acid substitutions in the gyrase subunit A (*Gyr*A) and *Par*C genes (Zhao & Zhao, 2012). Azithromycin (AZT) can become inactive due to mutations in the four copies of the 23S rRNA, production of methylase enzymes encoded by acquired genes (e.g., *erm*B/F),

or substitutions in the L4 and L22 ribosomal proteins (Chisholm *et al.,* 2010). Tetracycline (TET) is usually ineffective because of the tetracycline subunit M (*tet*M) gene acquisition (Isco & Alexander, 2011). Resistance to penicillin (PEN) is due to the production of acquired TEM-1-like β-lactamases or alterations of the penicillin binding proteins (PBPs; e.g., amino acid substitutions for PBP1 and PBP2) (Ison & Alexander, 2011). Notably, the over-expression of the multiple transferable resistance (*MtrCD*E) efflux pumps due to substitutions in its repressor *mtrR* or deletions/insertions in the *mtr* promoter region may reduce susceptibility to all of the above mentioned drugs which are azithromycin, ciprofloxacin and penicillin (Warner *et al.,* 2008), whereas substitutions in the *por*B outer membrane porin gene may affect PEN and TET antimicrobials (Allen *et al.,* 2011). However, the most threatening global concern is represented by the emergence of *N. gonorrhoeae* isolates resistant to cefixime (CFX) and, even more importantly, to ceftriaxone (CRO) (Camara *et al.,* 2012). Reduced susceptibility to these cephalosporins can be due to a mixture of the mechanisms involved in penicillin resistance, but full resistance necessitates specific mosaic structures of the PBP2 that are caused by recombination events occurring between gonococcus and other commensal *Neisseria* species (Ohnishi *et al.,* 2011).

The most common mechanism in gonococci for decreased ESC susceptibility is alteration of the *pen*A gene, i.e., the acquisition of a *pen*A mosaic allele or alterations of amino acid A501 in the encoded penicillin-binding protein 2 (PBP2) (Barry & Klausner, 2009). Furthermore, specific *por*B1b mutations that alter amino acid G101 and A102 in the PorB1b porin (the *pen*B resistance determinant) result in additional decreases in ESC susceptibility (Tomberg *et al.,* 2010). Nevertheless, based on the relatively few studies of gonococcal isolates with decreased ESC susceptibility, polymorphisms in *pon*A (encoding PBP1) and *pil*Q (encoding the pore-forming secretin PilQ protein in the type IV pili), which both can be involved in high-level penicillin resistance, do not seem to substantially enhance the MICs of ESCs (Golparian *et al.,* 2010).

2.4 *Chlamydia trachomatis*: Classification of the organism

Chlamydiae bacteria are obligate intracellular bacteria that were first thought to be protozoa and later a virus, but it became clear that *Chlamydiae* had all the important features of bacteria (Kuo & Stephens, 2011). *Chlamydiae* are placed in the order, *Chlamydiales,* with one family *Chlamydiaceae* which contain two genera, *Chlamydia* and *Chlamydophila* (Kuo & Stephens,

2011) (Table 2.3). Members of the genera are separated based on distinct clustering of the 16S rRNA gene sequences (Kuo & Stephens, 2011).

The family of bacteria has representatives extensively distributed in nature (Kuo & Stephens, 2011). The most important human species are *Chlamydia trachomatis, Chlamydopila pneumoniae* and *Chlamydophila psittaci* (Darville, 2005). The distinguishing characteristics between these species range from clinical presentation and antibiotic susceptibility, the staining characteristics (due to glycogen inclusions), inclusion morphology, shape of the elementary body and limited DNA sequence homology (Darville, 2005).

Table 2.3: Scientific classification of *C. trachomatis* (Euzéby, 2012)

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

Chlamydia trachomatis is divided into subgroup based on antigenic variation in the major outer membrane protein (MOMP) (19 serovars) and on clinical expression (biovars) (Joubert & Sturm, 2011). Analysis of clinical disease and the plasticity zone suggests that *C. trachomatis* should be grouped into lymphogranuloma venereum (LGV) biovars (serovars L1-L3) and the trachoma biovars (serovars A-C) (Joubert & Sturm, 2011). Seventy percent of non-LGV STIs are due to serovars D and E (Joubert & Sturm, 2011).

2.4.1 General characteristics of *Chlamydia trachomatis*

Chlamydia trachomatis is a non-motile, Gram-negative obligate intracellular bacterium (Hogan *et al.,* 2004). It has a double cell wall but lacks a peptidoglycan layer (Hogan *et al.,* 2004). *Chlamydia trachomatis* is slow growing and rarely exchanges DNA with other related *Chlamydiae* (Hogan *et al.,* 2004). The central metabolic pathways of *C. trachomatis* are aerobic, with glutamates as the primary carbon source supplemented by glucose (Carlson *et al.,* 2005). *Chlamydia trachomatis* is characterised by an unique two phase life cycle, that

alternate between the elementary body (EB) and the reticulate body (RB) (Hogan *et al.,* 2004). The EB is the metabolically inactive form that acts like fungal spores and are responsible for transmission of infection while the RB is the metabolically active form which is responsible for intracellular replication (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.,* 2011). The genomes of the representatives of all four serovars show an extremely high degree of conservation (Albrecht *et al.,* 2011). All biovars has a small and highly conserved genome of approximately 1 000 kb in size (1 042 519 bp) and contains both RNA and DNA (Carlson *et al.,* 2005; Albrecht *et al.,* 2011). The sequenced genome contains a 58.7% A+T nucleotide content, 7 493 bp of plasmids, 894 protein coding sequence (CDS) and a difference of 5 000 bp between the biovars (Albrecht *et al.,* 2011). The cryptic plasmid which is a small DNA molecule within a cell that is physically separated from a chromosomal DNA and can replicate independently, carries non-coding RNAs and eight conserved plasmid open reading frames (ORFs) deletion mutants designated (Albrecht *et al.,* 2011). 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 and may indicate an increased biological fitness of nvCT (Albrecht *et al.,* 2011).

2.4.2 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 (Byrne, 2010).

Chlamydia trachomatis has a complex mechanism for host cell entry and attachment (Cornelis & van Gijsegem, 2000). The type III secretion system is a complex arrangement of structures that are designed to promote delivery of pathogen affector proteins after contact with the cell (Cornelis & van Gijsegem, 2000).

The cytotoxin gene arrangements have been useful in defining *C. trachomatis* disease phenotype in that genital biovars contains a single gene with a large central deletion and LGV strains lack the toxin gene (Byrne, 2010). The *C. trachomatis* genome encodes proteins with significant homology to the large clostridia toxin B protein (Byrne, 2010). This cytotoxin produces morphological and cytoskeletal damage to the epithelial cells (Byrne, 2010). This effector protein accelerates the infection process (Byrne, 2010).

Essentially, the chlamydia life cycle constitutes its pathogenic process. During invasion of host cells, the EB enters the mucosal cell and induces its own endocytosis, then mature to form a RB which undergoes binary fission (Manavi, 2006). After the incubation period and multiple divisions by binary fission, the RB differentiates into the EB development stage and the infectious EB are released to initiate new rounds of infection through exocytosis (Figure 2.2) (Manavi, 2006).

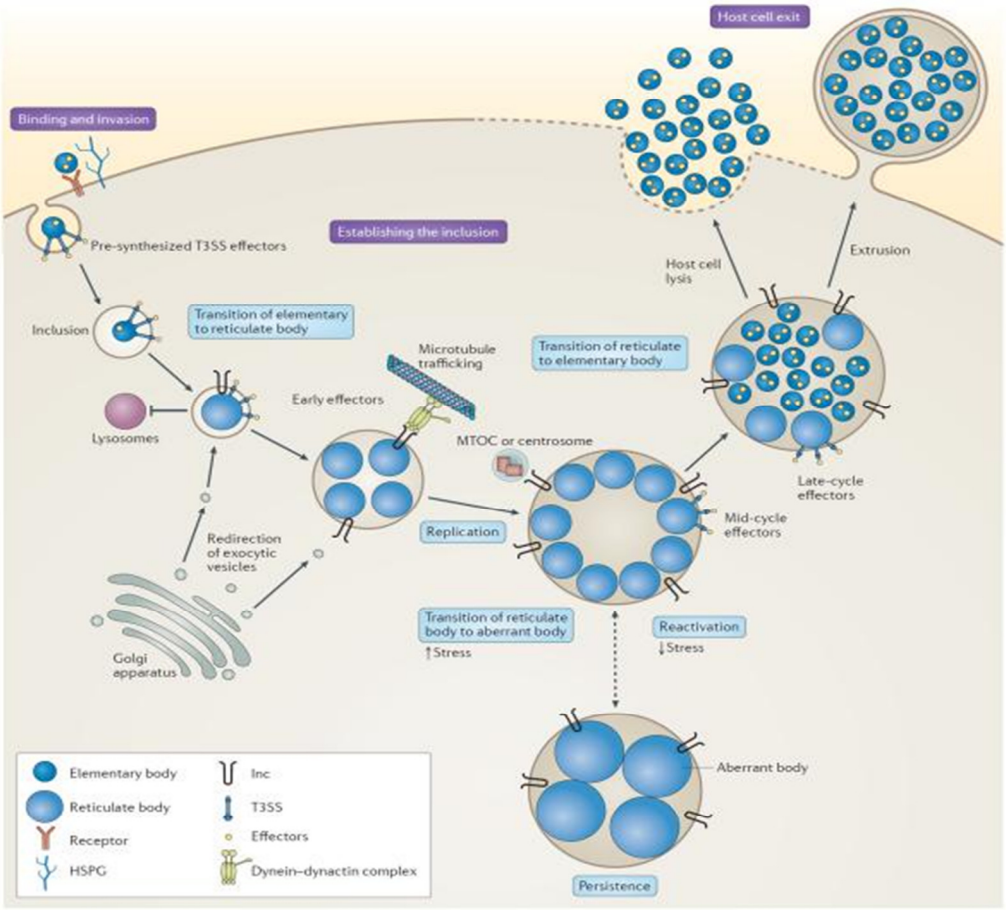

Figure 2.2: The life cycle of *Chlamydia trachomatis* (Manavi, 2006)

2.5 Bacterial vaginosis

Bacterial vaginosis (BV) is the most common cause of vaginal discharge in women (Allsworth & Peiper, 2007). It is characterised by the depletion of hydrogen peroxide (H_2O_2)-producing lactobacilli that characterise the normal vaginal flora and overgrowth of facultative (*Gardnerella vaginalis*) and anaerobic bacteria (*Prevotella* species, *Mycoplasma hominis, Bacteroides* spp., *Peptostreptococcus* spp., *Fusobacterium* spp., *Mobiluncus spp*., *Atopobium vaginae*, etc.) (Hillier *et al.,* 1993).

One in three women in the U.S. has bacterial vaginosis (BV), a microbial imbalance of the vaginal flora characterised by the absence of normally dominant lactobacilli and an overgrowth of complex communities dominated by Gram-negative bacteria and *Actinobacteria* (Srinivasan *et al.,* 2010). The study of Redelinghuys *et al.* (2015) conducted in Pretoria, South Africa, reported 17.7% (39/220) women having BV flora while 15% women had intermediate vaginal flora (IVF). Bacterial vaginosis can be asymptomatic, maybe even part of a spectrum of 'normal' from the patient's perspective, but often displaying characteristic clinical features, including ''thinning'' of vaginal fluid secretions, increased pH (>4.5), a fishy odour upon potassium hydroxide treatment and the presence of clue cells (epithelial cells studded with bacteria) in wet mounts (Srinivasan *et al.,* 2010). An additional defining feature of BV is the presence of vaginal sialidase, an enzyme that cleaves terminal sialic acid residues from complex glycans, which are abundant on host cell surfaces and secreted mucus proteins (Srinivasan *et al.,* 2010). Women with BV are at increased risk of pelvic inflammatory disease, infections following surgery or other routine gynecologic procedures, sexually transmitted infections including HIV, and serious pregnancy complications such as intrauterine infection and preterm birth (Marrazzo *et al.,* 2010).

2.5.1 The composition and structure of the vaginal microbiota

Comprehensive surveys of vaginal microbial communities using culture-dependent approaches have revealed that *Lactobacillus* species are the dominant vaginal bacterial species in the majority of women (Ma *et al.,* 2012). However, an appreciable proportion of asymptomatic otherwise healthy individuals have vaginal microbiota lacking significant numbers of *Lactobacillus* spp. and harbour a diverse array of facultative and strickly anaerobic microoganisms (Ma *et al.,* 2012).

Members of the genus *Lactobacillus* are commonly identified as the hallmark of normal or healthy vagina (Donders *et al.,* 2000). *Lactobacillus* spp. are thought to play a major role in protecting the vaginal environment from non-indigenous and potentially harmful microorganisms (Alakomi *et al.,* 2000). This is accomplished through the production of lactic acid, resulting in a low and protective pH (3.5 to 4.5) (Alakomi *et al.,* 2000). Interestingly, lactic acid has been shown to be more effective than acidity alone as a microbicide against HIV or against pathogens like *N. gonorrhoeae* (Graver & Wade, 2011). A study using *in vitro* colonisation of vaginal bacterial cell monolayers with common bacteria, such as *L. crispatus, Prevotella bivia* and *Atopobium vaginae,* demonstrated that these key vaginal bacteria appear to regulate the epithelial innate immunity in a species-specific manner (Fichorova *et al.,* 2011).

Lactobacillus crispatus was previously thought to be one of the most common species of lactobacilli in the vagina (Antonio *et al.,* 1999). However, the application of culture-independent methods has identified *L. iners,* an organism that is difficult to cultivate and which does not grow on traditional culture media, as the most prevalent vaginal bacterial species (Zhou *et al.,* 2004). In the studies by Burton *et al* (2003) and Zhou *et al* (2004), vaginal microbiota of 42% and 66% of the reproductive age women sampled were dominated by *L. iners* respectively. A large-scale cross-sectional study of 396 healthy asymptomatic women revealed that *L. iners* was detected in 83.5% of the subjects and dominated 34.1% of the vaginal bacterial communities analysed, while *L. crispatus, L. gasseri* and *L. jansenii* were present in 64.5%, 42.9% and 48.2% of the subjects and dominated in 26.2%, 6.3% and 5.3% of the vaginal bacterial communities respectively (Ravel *et al.,* 2011). This large study showed that vaginal bacterial communities that had similar species composition and abundance could be classified into five groups, which are refered to as community state type (Ma *et al.,* 2012). Studies have found that 20% to 30% of asymptomatic otherwise healthy women harbour vaginal communities that lack appreciable numbers of *Lactobacillus* but include a diverse array of facultative or strickly anaerobic bacteria that are associated with a somewhat higher pH (5.3 to 5.5) (Ravel *et al.,* 2011). The microbiota include members of the genera *Anaerococcus, Atopobium, Corynebacterium, Eggerthella, Finegoldia, Gardnerella, Mobiluncus, Peptoniphilus, Prevotella* and *Sneathia* (Ravel *et al.,* 2011).

2.5.2 Polymicrobial nature of bacterial vaginosis

Unlike most common infectious diseases, BV appears to be polymicrobial in nature (Gajer *et al.,* 2012). Genomic studies have illustrated the complexity and heterogeneity of BV, which can vary in bacterial composition from day to day and from one individual to another (Gajer *et al.,* 2012). Although more than a dozen bacterial species have been associated with BV, the potential causal contributions of each to the biochemical, cellular and clinical features of BV remains elusive (Ravel *et al.,* 2011). Microbiologically, this condition is characterised by a dramatic shift of vaginal microflora which involves the loss of beneficial bacteria (lactobacilli) and a simultaneous proliferation of anaerobic bacteria including *G. vaginalis, Atopobium vaginae, Mobiluncus* spp., *Bacteroides* spp., and *Prevotella* spp. (Verhelst *et al.,* 2004). *Gardnerella vaginalis* was the first bacterium implicated in the pathogenesis of BV and continues to be associated with the disease (Linhares *et al.,* 2010). However, there has been much debate in the literature concerning the contribution of *G. vaginalis* to the development and pathogenesis of BV (Linghares *et al.,* 2010). *Gardnerella vaginalis* is a facultative anaerobic, catalase and oxidase-negative bacterium. *Gardnerella vaginalis* cells are pleomorphic, Gram-negative to Gram-variable, non-encapsulated and non-motile rods with a mean size of 0.5 µm to 1.5 µm (Linghares *et al.,* 2010). *G. vaginalis* can be isolated/ detected from asymptomatic women that do not meet the criteria for BV diagnosis at the time of detection, raising questions regarding its potential role in BV (Gelber *et al.,* 2008). However, consistent with the notion of *G. vaginalis* as a potential pathogen, strains identified as *G. vaginalis* have been isolated from invasive perinatal infections (Gelber *et al.,* 2008). Moreover, several investigations have described the pathogenic potential of some *G. vaginalis* isolates in cell adhesion and entry, cytolytic toxin production, biofilm formation and other phenotypes that may reflect virulence (Gelber *et al.,* 2008; Zozaya-Hinchliffe *et al.,* 2010).

2.5.3 Diagnosis of bacterial vaginosis

The most frequently used method for BV diagnosis is the physician's assessment by the Amsel clinical criteria (Amsel *et al.,* 1983). This method is fairly subjective and is based on the observation of the following symptoms (i) vaginal fluid with a pH above 4.5; (ii) a positive "whiff test" (detection of fishy odour upon 10% potassium hydrogen addition); (iii) presence of clue cells (vaginal epithelial cells covered by bacteria) on microscopic examination of vaginal fluid and (iv) homogeneous milky vaginal discharge (Amsel *et al.,*

1983). At least three of the four symptoms described above must be present to establish a positive BV diagnosis (Workowski & Berman, 2006). Despite the fact that the Amsel's criteria do not require intensive training, it is not the most appropriate method to diagnose BV, due to its low specificity (Dickey *et al.,* 2009). Laboratory diagnosis is based on the Nugent score analysis, a microscopic method that quantifies three different bacterial morphotypes present in the vaginal smears (Nugent *et al.,* 1991). Nugent and colleagues (1991) have created a Gram stain scoring system based on the evaluation of the following morphotypes: (i) large Gram-positive rods (*Lactobacillus* spp. morphotypes); (ii) small Gram-variable rods (*G. vaginalis* morphotypes); (iii) small Gram-negative rods (*Bacteroides* spp. morphotypes); and (iv) curved Gram-variable rods (*Mobiluncus* spp. morphotypes). Each morphotype is quantified from 0 to 4 with regard to the number of morphotypes observed in the microscopic fields of the Gram-stained vaginal smear. The vaginal microflora is classified in normal microflora (scores of 0 to 3) or as BV (scores of 7 to 10), based on the sum of each morphotype score (Nugent *et al.,* 1991). Furthermore, due to its low specificity, the Nugent score system also considers intermediate microflora whenever the final score is between 4 and 6.

Although both methodologies are easy and fast to perform, the methods do not provide a robust diagnosis of BV (Machado *et al.,* 2015). When combined, these standard tests have a sensitivity and specificity of 81% and 70% respectively (Forsum *et al.,* 2005). To improve BV diagnosis, several new molecular methodologies have been proposed, with fluorescence *in situ* hybridisation (FISH) being a very promising alternative (Justé *et al.,* 2008). This technique combines the simplicity of microscopic observation and the specificity of DNA/rRNA hybridisation, allowing the detection of selected bacterial species and morphologic visualisation (Justé *et al.,* 2008). Peptide nucleic acid (PNA) probes are used instead of natural nucleic acids to improve the efficiency of FISH because these probes enable faster and more specific hybridisation (Peleg *et al*., 2009). In the design of PNA probes, the bases are linked by a neutral peptide backbone to avoid the repulsion between the negatively charged phosphate backbones which is observed in DNA/DNA hybridisation assays (Stender *et al*., 2002). In addition, the hybridisation step can be performed more efficiently in a low salt concentration, which promotes the destabilization of rRNA secondary structures and consequently improves the access to target sequences (Almeida *et al.,* 2009). All these advantages have made PNA-FISH a promising tool for diagnosis and therapy-directing

techniques, providing a rapid and accurate diagnosis of several microbial infections (Shepard *et al*., 2008; Trnovsky *et al.,* 2008).

2.6 What is the importance of genital mycoplasmas?

Preterm birth is one of the main causes of perinatal morbidity and mortality (Kramer *et al.,* 2000). *Ureaplasma urealyticum* and *Mycoplasma hominis* are commonly isolated from amniotic fluid in patients with spontaneous preterm labour or preterm premature rupture of membranes (PPROM) (Patel & Nyirjesy, 2010). Most studies have consistently demonstrated that the presence of genital mycoplasmas in amniotic fluid or placental membranes is associated with adverse pregnancy outcomes; however, the role of genital mycoplasmas is controversial (Patel & Nyirjesy, 2010). Some investigators have reported that women carrying vaginal *U. urealyticum* have a high risk of preterm birth or PPROM, but prospective studies have not shown a consistent association between vaginal *U. urealyticum* and preterm birth (Patel & Nyirjesy, 2010). Although numerous studies have reported the clinical significance of *U. urealyticum* in vaginal fluid, only a few studies have investigated the risk associated with *M. hominis* (Choi *et al.,* 2012; Capoccia *et al.,* 2013). *Mycoplasma genitalium* has been identified as a possible cause of pelvic inflammatory disease (Haggerty *et al.,* 2008). Furthermore, the separation of certain *Ureaplasma* organisms into a distinct species, *Ureaplasma parvum*, has spurred new questions about the role of ureaplasmas in causing female genital tract disease (Haggerty *et al.,* 2008).

2.6.1 General charactistics of genital mycoplasmas

Mycoplasmas are one of the smallest and simplest prokaryotes (*Tenericutes*), having only the minimal cellular machinery required for self-replication and survival (Thompson *et al.,* 2010). These bacteria appear to have evolved from Gram-positive bacteria by a process of degenerative evolution towards genome reduction and the loss of a cell wall (Thompson *et al.,* 2010). Mycoplasmas are widespread in nature as parasites of arthropods, fish, humans, mammals, plants and reptiles (Thompson *et al.,* 2010). In addition, mycoplasmas have been a problem as intracellular contaminants in human cell therapy and in animal (poutry and swine farming) production as pathogens (Thompson *et al.,* 2010).

2.6.2 Molecular diagnosis of genital mycoplasmas

Phenotypic and genotypic methods for the identification of mycoplasmas are available. Culture is still regarded as the gold standard for the detection of recoverable bacteria like *M. hominis* and *Ureaplasma* spp.; however, a low sensitivity when compared to PCR assays have been reported (Zeighami *et al.,* 2007). Culture is labour intensive and time consuming as it requires the use of an enrichment broth for up to seven days, followed by sub-culturing on solid media (Cao *et al.,* 2007). Analytical sensitivities in the range of 60% are only obtained in skilled laboratories and identification is restricted to genus level (Zeighami *et al.,* 2007). The development of commercially available diagnostic assays, which are based on liquid broth culture, provides easy to use and faster alternatives to conventional culture methods for the detection of genital mycoplasmas (Tarrant *et al.,* 2009). The difficulty of laboratory culture methods to isolate *M. genitalium* complicates antimicrobial susceptibility testing (Edberg *et al.,* 2008). There is currently no approved commercially available diagnostic assay for the detection and antimicrobial susceptibility testing for *M. genitalium*; detection is mainly done by nucleic acid amplification tests (Lillis *et al.,* 2011).

The commercially available Mycofast Revolution (EliTech Diagnostic, France) kit is a CE approved assay (European Confirmatory; A mandatory European marking for certain product groups to indicate conformity with the essential health and safety requirements set out in European Directive) (CLSI, 2011). This assay provides easy identification and enumeration for *M. hominis* and/or *Ureaplasma* spp. within 24 h to 48 h (CLSI, 2011). The Mycofast Revolution assay is a liquid method based on the ability of *Ureaplasma* spp. and *M. hominis* to metabolize urea and arginine, respectively and consists of 20 wells that are pre-coated with a dehydrated culture medium (foal serum, yeast extract, cysteine, arginine, urea, phenol red and antibiotics) and contains a single broth with antimicrobials for transport and preservation of genital mycoplasmas (UMMt) (ELiTech Diagnostic, France) (CLSI, 2011). The advantage of the Mycofast Revolution assay is that antimicrobial susceptibility testing is performed against different antimicrobial agents with specific minimum inhibitory concentrations (MICs) as defined by the 2011 Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2011). Antimicrobial susceptibility testing is performed for five antimicrobial agents including clindamycin, erythromycin, levofloxacin, moxifloxacin and tetracycline (CLSI, 2011).

Molecular methods, such as PCR assays are reported to be more sensitive for diagnostic purposes than culture (Waites *et al.,* 2012). Waites *et al*. (2012) indicated that PCR-positive, culture-negative specimens are likely to represent true positives due to the much higher sensitivity. Polymerase chain reaction is a laboratory technique for DNA replication that allows a target DNA sequence to be selectively amplified. Genotypic methods also allow for speciation, which is a limitation of culture methods (Waites *et al.,* 2012). Other advantages include the rapid detection as well as that these assays do not rely on the viability of the bacterium for detection (Gdoura *et al.,* 2008). Furthermore, when using a multiplex PCR (mPCR) assay, the detection of more than one target in a single reaction is possible and this can simplify the workflow (Mclver *et al.,* 2009).

2.7 Diagnosis of sexually transmitted infections

It is important to increase vigilance and to recognize that STIs are often asymptomatic or cause nonspecific symptoms and that a STI can increase the infectiousness of HIV and that regular testing for STIs is crucial (Celum, 2010). The female genital tract represents a highly dynamic environment with resident microflora consisting of a variety of different species, such as *Lactobacillus* spp. and *Atopobium vaginae* (Diaz *et al.,* 2010). The co-existence of different sexually transmitted microorganisms is a common event; it is due to several factors, such as (i) common route of transmission, (ii) the host's sexual behaviour and (iii) the resident flora (Diaz *et al.,* 2010). Nevertheless, little attention has been dedicated so far to the presence of co-infections in the human vagina and to their clinical and diagnostic implications (Diaz *et al.,* 2010). Scientific progress has provided a broad array of tests for identification of STIs (Unemo *et al.,* 2013). These tests vary greatly in terms of their level of complexity (i.e. the technical requirements for optimal test performance), in the costs required to perform them (both material- and labour-related), and in terms of performance. Culture, antigen detection, or nucleic acid detection using either amplified or non-amplified nucleic acid detection tests are often more sensitive than microscopy but may have more complex technical requirements for optimal test performance and may increase the interval between testing and the availability of test results (rapid POC tests help to overcome the latter potential limitation).

2.7.1 Phenotypic

Microscopic examination of a wet mount preparation of vaginal secretions mixed with normal saline is the most common diagnostic evaluation for *T. vaginalis* infection in women (Huppert *et al.,* 2007). Direct observation of the pear-shaped trichomonads with their characteristic jerky or tumbling motility is considered 100% specific for *T. vaginalis* (Huppert *et al.,* 2007). Delays that is as short as 10 to 30 min between specimen collection and microscopic examination can dramatically reduce the sensitivity of the test (Huppert *et al.,* 2007). Culture techniques include the use of Diamond's modified medium, available in glass tubes from commercial microbiological media suppliers, and the InPouch TV test (Biomed Diagnostics, Oregon, USA) (Patil *et al.,* 2012). The InPouch TV test is a self-contained culture pouch made of oxygen-resistant, optically clear plastic that can be microscopically examined directly, eliminating the need to remove material from the culture for daily examination (Patil *et al.,* 2012). The InPouch TV culture media has the following advantages: (i) ease of use as two procedures are combined into one test; (ii) it has a long-shelf life; (iii) no expensive equipment is required and (iv) cultures can be obtained even with a very low concentration of trichomonads (Patil *et al.,* 2012).

The OSOM (formerly Xenostrip) *Trichomonas* Rapid Test (Sekisui Diagnostics, Framingham, MA) is an immunochromatographic capillary-flow dipstick test and the only rapid antigen test commercially available in the US (Huppert *et al.,* 2007). It is performed on vaginal secretions with results available within 10 min. This point-of-care test is FDA-cleared for females and Clinical Laboratory Improvement Amendments (CLIA) waived (Huppert *et al.,* 2007). Test specifications include sensitivity of 82% to 95% and specificity of 97% to 100% (Campbell *et al.,* 2008). A rapid point-of-care test either alone or in combination with wet mount microscopy, may be considered alternatives to wet mount microscopy alone in settings in which microscopy is available (Jones *et al.,* 2013).

Because of its high specificity (>99%) and sensitivity (>95%), a Gram-stain of a male urethral specimen that demonstrates polymorphonuclear leukocytes with intracellular Gram-negative diplococci can be considered diagnostic for infection with *N. gonorrhoeae* in symptomatic men (Elias *et al.,* 2011). Gram stains of endocervical specimens; pharyngeal or rectal specimens also are not sufficient to detect infection and therefore are not recommended (Elias *et al.,* 2011). It is important to specifically test for *N. gonorrhoeae* as highly sensitive and

specific testing methods are available and because a specific diagnosis might enhance partner notification (Elias *et al.,* 2011). Specimens collected for gonorrhoea culture should be obtained by using swabs with plastic or wire shafts and rayon, Dacron, or calcium alginate tips. Other swab material, such as wood shafts and cotton tips might be inhibitory or toxic to the organism and should be avoided (Drake *et al.,* 2005).

Specimen collection for *C. trachomatis* culture is invasive requiring insertion of a swab 2 cm to 3 cm into the male urethral or 1 cm to 2 cm into the endocervical canal followed by two or three rotations to collect sufficient columnar or cuboidal epithelial cells (Watson *et al.,* 2002). The specimen is inoculated by centrifugation onto a confluent monolayer of McCoy, HeLa 229, or Buffalo green monkey kidney cells that support growth of *C. trachomatis* (Watson *et al.,* 2002). Once the specimen has been inoculated, 2 μg/mL of cycloheximide should be added to the growth medium to suppress protein synthesis by the host eukaryotic cell (Watson *et al.,* 2002). Inoculated cells are harvested after 48 h to72 h of growth; infected cells develop characteristic intracytoplasmic inclusions that contain substantial numbers of *C. trachomatis* elementary and reticulate bodies (Watson *et al.,* 2002).

2.7.2 Genotypic: Molecular diagnosis of STIs

Nucleic acid amplification tests (NAATs) are the most sensitive tests available for detection of *T. vaginalis* (Hobbs & Séna, 2013). Nucleic acid amplification tests are also available as fully automated closed systems that can be run independently of centralized laboratories and will become increasingly important for point-of-care testing (Brockmeyer & Meyer, 2016). The APTIMA *Trichomonas vaginalis* assay (Hologic Gen-Probe, San Diego, CA) was FDA-cleared in 2011 for use with urine, endocervical and vaginal swabs and endocervical specimens collected in the Hologic Preserve Cyt solution (ThinPrep) from females only (Hobbs & Séna, 2013). Sensitivity is 95% to 100% and specificity is also 95% to 100% (Hobbs & Séna, 2013). The BD ProbeTec *Trichomonas vaginalis* Qx Amplified DNA assay (Becton Dickinson, Franklin Lakes, NJ) launched in Europe (EU cleared) in 2012 is not FDA-cleared in the US (Hobbs & Séna, 2013).

Nucleic acid amplification tests have high sensitivity and specificity compared to culture for the detection of *N. gonorrhoeae* (Shipitsyna *et al.,* 2008). The main types of NAATs that are used for the detection of *N. gonorrhoeae* include transcription-mediated amplification (TMA),

PCR, real-time nucleic acid sequence based amplification (NASBA) and strand displacement amplification (SDA) (Shipitsyna *et al.,* 2008; Le Roux *et al.,* 2010; Walker & Sweet, 2011). All these methods detect and copy gonococcal DNA to enhance detection (Walker & 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 and vaginal swabs by the US FDA (Walker & Sweet, 2011). The disadvantages of NAATs include the high cost and the risk of cross contamination with highly sensitive amplification methods (Walker & Sweet, 2011).

The first molecular DNA test for *C. trachomatis* was a nucleic acid hybridization test (NAH) database probing (Bébéar & De Barbeyrac, 2009). One commercially available NAH test is PACE 2 (Gen-Probe Inc, USA). 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.,* 2006). 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 is more sensitive (90% to 95%) and specific (99% to 100%) compared with cell culture because it amplifies specific DNA and RNA target sequences unique to *C. trachomatis* in clinical specimens (Schachter *et al.,* 2005). New assays and new platforms that provide results at the time of the patient visits are available, since the patients do not return for their results and one of the platforms in Cepheid GeneXpert. The Cepheid GeneXpert assay is a rapid NAAT assay that can be perfomed on the site of the laboratories and it can be used as point-of-care platform (Gaydos *et al.,* 2013).

Since the consequences of genital infections through sexually transmitted disease can be as severe as sterility and disseminated infections, molecular assays detecting bacterial pathogens, such as *Chlamydia trachomatis* and *Neisseria gonorrhoeae*, were shown to be relevant not only in the diagnosis of infections but also in sexually active women who are presenting with few symptoms (Bignel & Unemo, 2013; Papp *et al.,* 2014). Several commercially available real-time PCR assays enable the detection of bacterial pathogens such as Anyplex II STI-7 PCR and Preto^{plus} CT/NG/TV assay (Tabrizi *et al.,* 2011). The Anyplex II STI-7 kit (STI-7, Seegene, Korea) is a multiplex real-time PCR assay relying on a newly developed tagging oligonucleotide cleavage and extension technology (TOCE) (Berçot *et al.,* 2015). This assay is marketed to simultaneously detect seven microorganisms involved in secually transmitted

infections: *C. trachomatis, N. gonorrhoeae, T, vaginalis, M. genitalium, M. hominis, U. urealuticum* and *U. parvum* and have a sensitivity of 100% and specificity of 99% (Berçot *et al.,* 2014). The Preto^{plus} CT/NG/TV assay (Microbiome Ltd, Houten, The Netherlands) is a triple detection assay which can be used on a variety of open PCR systems (de Waaij *et al.,* 2015). The principle of this test include the detection of *C. trachomatis, N. gonorrhoeae* and *T. vaginalis* DNA by the PCR which is based on the amplification of a part of the *C. trachomatis* cryptic plasmid DNA, the opa genes of *N. gonorrhoeae* and part of the 2-kb repeated sequence of *T. vaginalis* using specific oligonucleotides. It has been compared with to the Roche cobas 4800 CT/NG assay and it showed comparable results (de Waaij *et al.,* 2015).

2.8 Typing methods for *T. vaginalis, N. gonorrhoeae* and *C. trachomatis*

Molecular typing studies consistently describe a two-type population structure for *T. vaginalis (*Meade & Carlton, 2013). *Trichomonas vaginalis* genotype I proved to be more genetically diverse and, therefore, presumably older than genotype II in evolutionary history (Meade & Carlton, 2013). Surprisingly, the two genotypes seem nearly equally prevalent across the globe, except for a predominance of type I in South Africa and type II in Mexico (Conrad *et al.,* 2012). *Trichomonas vaginalis* genotype I is more frequently infected with the pathogenic *T. vaginalis* virus (TVV) and type II has been associated with metronidazole resistance (Conrad *et al.,* 2012). A more robust technique, multilocus sequence typing (MLST) was first introduced in 1998 and identifies polymorphisms in approximately seven housekeeping genes through sequencing of PCR amplicons (Cornelius *et al.,* 2012). However, this MLST scheme requires culturing of patient material to ensure sufficient DNA load for testing (Cornelius *et al.,* 2012).

Methods for auxotyping *N. gonorrhoeae* isolates were first described in 1973, and there were a number of subsequent modifications (Fredlund *et al.,* 2004). Auxotypes of *N. gonorrhoeae* isolates are based on their different nutritional requirements for amino acids, purines, pyrimidines and vitamins (Bash *et al.,* 2005). This method has a low discriminatory ability, is time consuming and laborious, and requires a high level of technical expertise and interpretation (Dunning-Hotopp *et al.,* 2006). The determination of serovars may be important for a better understanding of host immune response and immune protection and ultimately in the development of gonococcal vaccines (Starnino *et al.,* 2009). Serovars are determined

using coagglutination techniques for detecting interactions between gonococcal antigens and panels of specific monoclonal antibodies (MAbs) (Starnino *et al.,* 2009). The disadvantages include suboptimal discriminatory ability compared to modern genotypic methods, reproducibility issues due to the subjective interpretation of results, low specificity of some specific monoclonal antibodies (MAbs) and the increasing prevalence of non-serotypeable strains and the emergence of new serovars over time due to the ongoing evolution of *por*B (Starnino *et al.,* 2009; Tapsall *et al.,* 2010). In India and Pakistan, gonococcal epidemiology has been explored in a few, mostly outdated studies, using traditional, low discriminatory phenotypic typing methods, such as antimicrobial susceptibility testing, auxotyping, plasmid profiling, and serotyping (Kulkarni *et al.,* 2012). Only a few minor studies have used genetic gel-based typing methods (e.g., restriction fragment length polymorphism of the whole genomic DNA, ribotyping, and Opa-typing (Khaki *et al.,* 2009). All these methods suffer from a lack of an objective reading and interpretation of results (compared to sequence-based methods), international standardisation, and a database for international inter-laboratory comparisons (Unemo *et al.,* 2011).

Genotypic methods based on DNA sequencing are internationally recommended, of which *N. gonorrhoeae* multi-antigen sequence typing (NG-MAST) or full- or extended-length *por*B gene sequencing are currently the best methods for fast, objective, portable, highly discriminatory, reproducible, typeable, and high-throughput characterisation of *N. gono*rrhoeae strains (Martin *et al.,* 2004). However, it is multilocus sequence typing (MLST), which targets several gonococcal housekeeping genes, that is ideally suited for long-term or global epidemiological (macroepidemiology) investigations, including the evolutionary relationship between resistant strains (Unemo and Dillon, 2011). The application of MLST to investigate the emergence of ceftriaxone resistance in *N. gonorrhoeae* has so far identified two key sequence types (STs), namely ST7363 and ST1901, both of which have been associated with the mosaic penicillin-binding protein 2 (PBP2) sequence that is considered a pivotal building block for ceftriaxone resistance (Onhishi *et al.,* 2011). The major limitation of MLST is that it requires DNA sequencing of seven housekeeping genes and is thus costly and time consuming (Unemo and Dillon, 2011). To circumvent this problem, a single nucleotide polymorphism (SNP)-based method, targeting informative SNPs in the same gonococcal MLST housekeeping genes was developed (Whiley *et al.,* 2013). The method is able to distinguish the majority of known gonococcal MLST types based on a 14 SNP profile and achieved this at 30% of the cost of traditional MLST (Whiley *et al.,* 2013).

Although various genotyping methods are available for typing *C. trachomatis*, only two published methods demonstrated the degree of resolution needed for molecular epidemiological studies. In 2007, Klint *et al*. (2007) described the MLST method for *C. trachomatis* that included five variable regions: *hctB*, CT058, CT144, CT172, and penicillin binding protein 2B (*pbpB)*. A second technique, a multilocus variable-number tandem-repeat (VNTR) analysis (MLVA) published by Pedersen *et al*. in 2008, combined *ompA* with three highly variable single repeats: CT1291, CT1299, and CT1335 (Pedersen *et al.,* 2008). Both techniques were evaluated by Ikryannikova *et al*. (2010) and a high discriminatory power for cultured samples was reported. A drawback of the techniques is that both methods consist of single PCRs, which might have a decreased sensitivity with clinical samples tested directly (Ikryannikova *et al.,* 2010).

2.9 Treatment

Syndromic Management (SM) is a multifaceted strategy for STI control that includes the recognition of symptoms by the patient and an effective treatment regime that comprehensively covers the possible aetiological agents for a defined syndrome, appropriate health seeking behaviour of infected individuals and recognition of syndromes by the health care worker (Figure 2.3) (National Department of Health, 2015). The syndromic approach uses clinical algorithms so designed that primary health care nurses in resource poor settings may arrive at an appropriate clinical diagnosis based on a patient's symptoms and clinical signs (Figure 2.3) (Ballard *et al.,* 2000). The clinical diagnosis is then linked with a predefined antimicrobial prescription in which drugs are advised that have shown efficacy against the different STI pathogens in clinical trials (Figure 2.3) (Moodley, 2002). The ultimate objective of the SM strategy is to reduce the load of STIs and this has gained importance due to its possible impact on HIV transmission (National Department of Health, 2015).

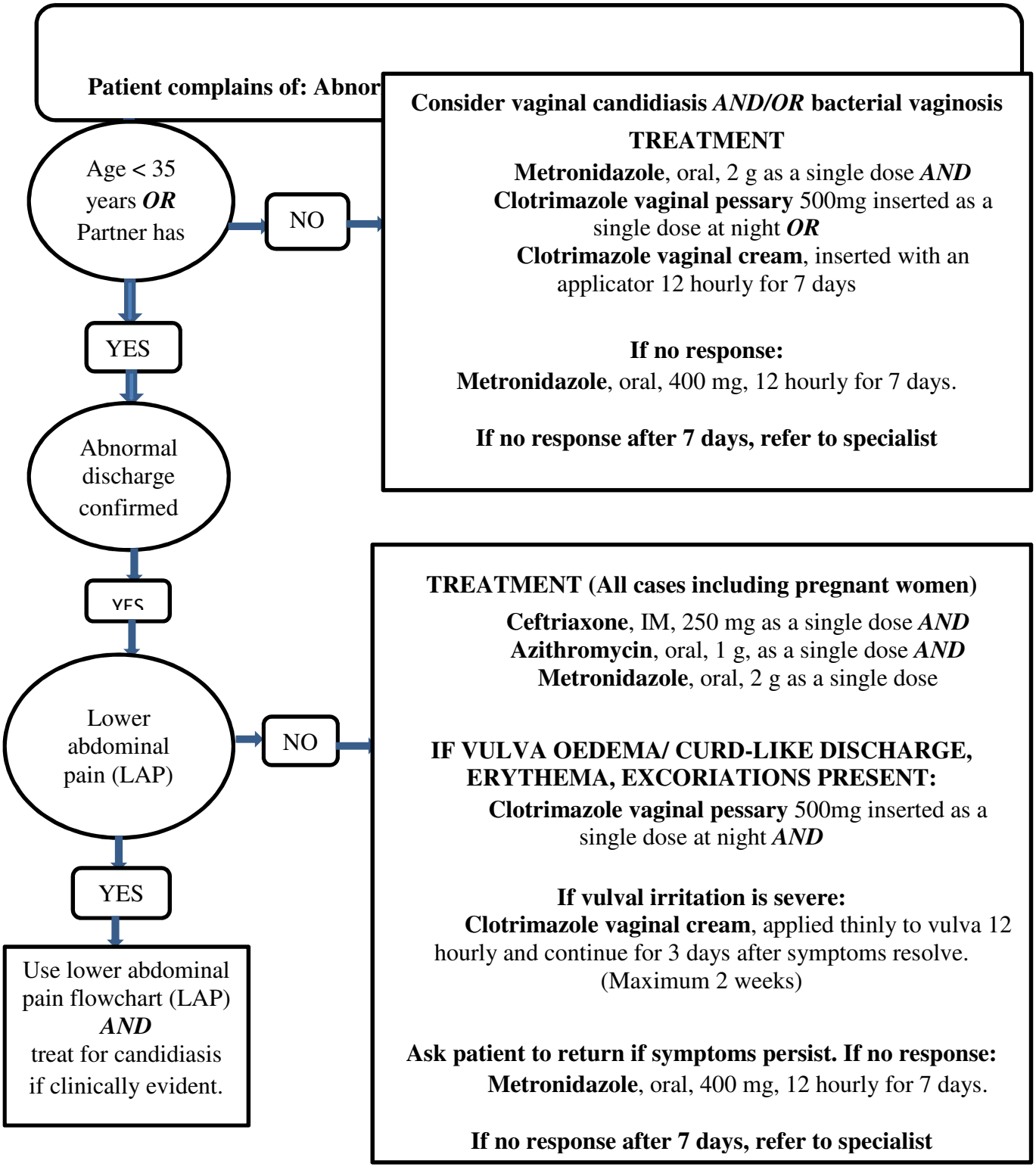

Figure 2.3: Syndromic treatment for vaginal discharge syndrome (National Department of Health, 2015)

The main advantages of the SM approach is that: (i) the patient receives effective treatment at the first visit because the treatment regime targets all common causative agents of the presenting symptoms; (ii) provides opportunity and time for education and counselling and (iii) the use of flow charts standardises diagnosis, treatment, referral and reporting, allowing for improved surveillance and programme management (Dallabetta *et al.,* 1998). Although much effort is being channelled into improving conditions in clinics and the quality of care, there are several problems that continue to recur or which are difficult to address given the limited resources the health sector is faced with (Dallabetta *et al.,* 1998). Several problems include; (i) Over-diagnosis and over-treatment which may result in increased drug costs, possible side-effects of multiple drugs, alterations in vaginal flora and the potential for increased drug resistance and (ii) the syndromic approach for vaginal discharge is poorly predictive of the presence of cervical chlamydial and/or gonococcal infection (Hawkes *et al.,* 1999).

2.10 Summary

Genito-urinaty infections, including STIs, are caused by a large number of different microbial agents that cause considerable morbidity and mortality worldwide (Amin *et al.,* 2007). Vaginal discharge syndrome and urethritis are the most common STI syndromes diagnosed in women and men (Mhlongo *et al.,* 2010). Urethritis occurs in both men and women but often is unrecognized in women (Mckechnie *et al.,* 2009). It is broadly categorised as non-gonococcal urethritis or gonococcal urethritis and it is characterised by discharge and dysuria (Tsai & Li, 2013). The pathogens responsible for vaginal discharge syndrome include *C. trachomatis* and *N. gonorrhoeae*, whilst the role of *M. genitalium* and *U. urealyticum* in these syndromes remains minimal (Mhlongo *et al.,* 2010).

The World Health Organization STI diagnostics initiative supports the development of cheap, reliable, non-invasive and easy-to-use tests, especially for the diagnosis of chlamydia and gonorrhoeae (Gaydos *et al.,* 2006). There are many different methods that can be used to detect *C. trachomatis, M. genitalium, N. gonorrhoeae* and *T. vaginalis*, which includes: (i) bacterial culture, (ii) enzyme-linked immunosorbent assays (ELISA) for antigen or antibody detection and (iii) molecular diagnostic assays (Chan *et al.,* 2000; Chernesky *et al.,* 2005; Muvunyai *et al.,* 2011). Molecular assays, such as PCR assays, transcription mediated amplification (TMA), etc. have been used to diagnose sexually transmitted pathogens in

clinical laboratories - PCR assays have been found to be highly sensitive for detecting these pathogens (Le Roux *et al.,* 2010; Muvunyai *et al.,* 2011). 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 (Muvunyai *et al.,* 2011). Furthermore, the inclusion of an internal control reaction in a multiplex assay identifies the possible presence of PCR inhibitions (Muvunyai *et al.,* 2011). Commercially available assays that are often used for simultaneous detection of sexually transmitted pathogens include; Anyplex II STI-7 detection real-time PCR, Cepheid CT/NG and TV Xpert rapid PCR, Roche cobas® 4800 real-time PCR and Presto^{plus} CT/NG/TV assays (Le Roux *et al.,* 2010; Gaydos *et al.,* 2013; Gueye *et al.,* 2014, de Waaij *et al.,* 2015).

The GeneXpert test is performed using a modular cartridge-based platform for testing each specimen by nucleic amplification and it can process from 1 to 96 specimens in less than 2 h with easy to use cartridges that minimise processing steps and contamination (Gaydos *et al.,* 2013). Cepheid GeneXpert assay's performance is highly accurate and reproducible and can be recommended for detecting *C. trachomatis, N. gonorrhoeae* and *T. vaginalis* in cervical, vaginal and urine specimens from women and in urine specimens from men (Gaydos *et al.,* 2013). The rapidity of the assay makes it valuable for clinic-based testing, as the results can be used to immediately treat infected patients in many cases (Gaydos *et al.,* 2013).

Good diagnostics are essential for prevention of further spreading of STI in the healthy population. Therefore diagnostic tests should display high sensitivity whereas false-positives have to be excluded at all time. Nucleic acid amplification tests are the most sensitive assays available to date for detecting STIs in clinical specimens and Xpert assays add to the group of commercially available assays that are available to laboratories as choices for superior diagnostic performance. The inability of Xpert to incorpate two *N. gonorrhoeae* targets of which would need to be positive for reporting a positive results, allows reporting with more confidence (Tabrizi *et al.,* 2011). The multiplex real-time PCR is cost saving because it can rapidly detect multiple microorganisms simultaneously through its multiplex function and it is expected that is will be used as a standard diagnostic test for STIs in near future.

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CHAPTER 3

TRICHOMONAS VAGINALIS AND BACTERIAL CO-INFECTION IDENTIFIED IN REPRODUCTIVE AGE WOMEN

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

3.1 Abstract

Sexually transmitted infections (STIs) continue to be a significant public health problem with an increased burden in women of reproductive age. According to a study conducted in 2011 for the South African Sentinel Surveillance data, the prevalence of STIs, such as *Trichomonas vaginalis*, *Chlamydia trachomatis* and *Neisseria gonorrhoeae*, has not declined in the eight years prior to 2011. *Trichomonas vaginalis* infections in women are mostly asymptomatic and it has been proven to be associated with adverse complications, such as preterm birth, pelvic inflammatory disease and infertility in reproductive age women. The aim of this study was to determine the prevalence of *T. vaginalis* and bacterial co-infections in reproductive age women. Sexually transmitted infections are associated with a wide spectrum of disease presentations, such as urethritis and epididymitis in men and cervicitis in women. Major STI pathogens detected in the study included *T. vaginalis* and its co-infections.

Methods: Self-collected vaginal swabs were obtained from participants vising two STI clinics, one being a public health and the other a private sexual health clinic. The swabs were analysed using the Nugent scoring system for bacterial vaginosis and molecular assays including GeneXpert CT/NG and TV PCR assay, Anyplex II STI-7 Detection PCR and a conventional PCR assay for the detection and identification of mycoplasmas.

Results:

The number of participants from a public health and a sexual private health clinic were 35 and 82 respectively. The prevalence of STIs increased from 12.5% in 2010 up to 13.7% in 2016. A high *T. vaginalis* prevalence differed by clinic population, with the highest detected in the private health clinic. High *T. vaginalis* numbers were also detected in the public health clinic. *Trichomonas vaginalis* infection occurred most often with *C. trachomatis* and *N. gonorrhoeae.* The co-infection rate was 2.6% (3/117) in this study. The prevalence rate of

T. vaginalis/ C.trachomatis co-infection was 12.5% (2/117) and *T. vaginalis/ N. gonorrhoeae* co-infection being 6.25% (1/117). Co-infection was common in women <30 years old of age. Being unmarried was significantly (p-value= 0.038) associated with the detection of a positive STI at both clinics.

Conclusion:

A high *T. vaginalis* prevalence was detected in this study which included asymptomatic reproductive women. The women screened for *C. trachomatis* and *N. gonnorrhoeae,* whether asymptomatic or symptomatic, should be screened for *T. vaginalis.*

3.2 Introduction

Since the discovery of *T. vaginalis* in 1836, the sexually transmitted obligate extracellular mucosal parasite has progressively grown from a perceived insignificant commensal to an important pathogen, inducing significant health sequelae in both men and women and with adverse pregnancy outcome (Hobbs *et al.,* 2008). *Trichomonas vaginalis* pathobiology includes its epidemiological association with human immuno-deficiency virus (HIV) over and above high-risk members of the population in terms of exposure to sexually transmitted infections (Johnston and Mabey, 2008). Individuals infected are often asymptomatic and without treatment, *T. vaginalis* may increase risks for conditions, such as pelvic inflammatory disease (Guenthner *et al.,* 2005).

Trichomoniasis can be considered the most common non–viral sexually transmitted infection in the world according to the WHO estimates, with an annual incidence of almost 250 million cases (WHO 2011). The incidence for *T. vaginalis* exceeded the incidence of *C. trachomatis* infections (101 million) as well as infection with *N. gonorrhoeae* (88 million) and *Treponema pallidum* (11 million) that occurs annually (WHO, 2011). *Trichomonas vaginalis* continues to be the most common curable sexually transmitted infection worldwide, with the regional prevalence rates in women ranging from 5% in the Western Pacific to 18% in Africa (Lewis *et al*., 2007; Poole & McClelland, 2013).

Between 2001 and 2004, US women aged 14 to 49 years participated in the National Health and Examination Survey (NHANES). Results from this national research study suggest that *T. vaginalis* is associated with the Latina and African-American ethnicity/race, increasing age

and poverty (Sutton *et al*., 2007). Among low income African-American adolescent women, studies revealed a 13% prevalence rate for *T. vaginalis* and that it was significantly associated with sex with non-steady partners (Crosby *et al.,* 2002). Cross-sectional studies have estimated *T. vaginalis* prevalence between 15% and 32% in a peripartum African cohort and an older age at sexual debut, higher education, being married and comorbid STIs have been all associated with prevalent *T. vaginalis* (Stringer *et al.,* 2010; Abdelaziz *et al.,* 2014). A study conducted in Durban, South Africa reported a *T. vaginalis* prevalence rate of 6.2% (217/3492) from women who are unmarried and not living with their partners (Naidoo & Wand, 2013).

Traditionally, the diagnosis of *T. vaginalis* has relied on microscopy and culture as the gold standard (WHO, 2011). However, most recently nucleic acid amplification tests (NAATs) using either urine or swab specimens have become the preferred methods of diagnosis due to greater sensitivity and specificity (WHO, 2011). Due to the distance from centralised laboratories and difficulties in recalling patients, 11% to 25% of patients diagnosed positive with a STI are left untreated and the average time to treatment is 21 days (Guy *et al.,* 2014). So the availability of an accurate portable molecular *T. vaginalis* point-of-care (POC) test, such as the GeneXpert platform (Cepheid, Sunnyvale, California, USA), may improve *T. vaginalis* screening and management and other low-resource settings where there is limited laboratory infrastructure (Badman *et al.,* 2016). The microbiological aetiology of STIs involves multiple pathogens and therefore, multiplex PCR provides an additional advantage in screening since it detects multiple pathogens simultaneously in a single clinical sample with high sensitivity and specificity (Samra *et al.,* 2011).

3.3 Methods

3.3.1 Study site and specimen collection

Three self-collected vaginal dry and one Amies medium (Copan Diagnostics, Inc, Italy) swabs as well as a GeneXpert (Cepheid, Sunnyvale, USA) swab were obtained from each participant attending a public health clinic and private health care clinic who are older than 18 and who gave informed consent. Exclusion criteria included women younger than 18 years of age and those that did not give informed consent. Inclusion criteria included all women of reproductive age (>18 years) who gave written informed consent. Demographic data such as HIV status, marital status and other relevant information were recorded in the demographic

data sheet for analysis purpose. All specimens were given unique laboratory numbers for confidentiality and processed in the Department of Medical Microbiology (University of Pretoria). Ethical approval (103/2015) was obtained from the Research Ethics Committee, Faculty of Health Sciences (University of Pretoria) prior to commencement of this study.

The first swab was placed in a 1 ml tube with transport medium (modified Amies transport medium) and left to immerse for 10 min for culturing. The second swab was used to prepare a smear by rolling the swab in a Z pattern onto a glass slide. The prepared smears were transferred to the laboratory for Nugent scoring. The third swab (in a media), Eswab (Becton Dickson, Sparks, USA) was transported to the laboratory for the real-time Anyplex STI-7 PCR assay. The fourth swab (in a media), GeneXpert (Cepheid, Sunnyvale, USA) was used for the GeneXpert real-time PCR assays. The results were made available to the clinician in case treatment was required.

3.3.2 Microscopy of Gram-stained vaginal smears

When evaluating Gram-stains for BV, three different morphotypes were evaluated: i) *Lactobacillus* species – large, sometimes long Gram-positive rods; ii) *Gardnerella vaginalis/Bacteroides* – small Gram-variable/Gram-negative rods (these two morphotypes are combined due to their similar appearance on Gram-stain) and 3) *Mobiluncus* – curved Gram-negative/Gram-variable rods. Five oil immersion fields per slide were examined for the presence of these three different morphotypes. Each was quantitated from 0 to 4+ with regard to the number of morphotypes per oil immersion field: 0 per oil immersion field = 0; <1 per oil immersion field = 1+; 1 to 4 per oil immersion field = 2+; 5 to 30 per oil immersion field = 3+; >30 per oil immersion field = 4+ (Table 3.1).

The Nugent score (a number between 0 and 10) was calculated by adding the assigned scores according to Table 3.1. A score of 0 to 3 was considered negative for bacterial vaginosis, a score of 4 to 6 was considered intermediate and a score of 7 to 10 was considered positive for bacterial vaginosis.

3.3.3 Detection and identification of *T. vaginalis* by culture and microscopy

One of the collected vaginal swabs (Copan Diagnostics, Inc, Italy) of some participants were placed into the InPouch chamber (Biomed Diagnostics, Oregon, USA) and agitated to release the adherent protozoans. The InPouches were examined under the microscope for *T. vaginalis* immediately upon receipt and the results were recorded. The InPouches were incubated in an incubator (Scientific Incubator Vacutec, Johannesburg, SA) with a regulated temperature of 37°C up to five days in 5% CO_2 environment while recording results each day. A diagnosis of *T. vaginalis* was made after the first positive pouch reading. After five negative pouch readings, the specimen was considered *T. vaginalis* culture negative. Positive *Trichomonas* protozoans under the microscope appear as being motile with a jerky movement and flagella can be seen.

3.3.4 Detection and identification of *N. gonorrhoeae*

The first collected vaginal swab was streaked over a small portion of the Thayer-Martin agar (Hardy Diagnostics, Santa Maria, USA) surface, and streaked all over for isolation on the medium. It was incubated in a 5% to 10% CO_2 incubator (Scientific Incubator Vacutec, Johannesburg, SA) at 35°C to 37°C for 24 h. Incubation was extended until growth was observed for a further 24 h. Subcultures of *N. gonorrhoeae* on Thayer-Martin (Hardy Diagnostics, Santa Maria, USA) media were made and incubated for 18 to 24 h for further investigation.

3.3.5 Molecular detection of bacterial sexually transmitted co-infections

The automated Xpert CT/NG assay (Cepheid, Sunnyvale, USA) was used to detect *C. trachomatis* and *N. gonorrhoeae* directly from the swab samples. The AnyPlex real-time PCR assay as well as a conventional PCR assay for detection of the mycoplasmas was done using extracted DNA from the swab samples.

3.3.6 Genomic DNA extraction of the swabs using a commercial kit

Genomic DNA was isolated from the second collected vaginal swab with the ZR Fungal/Bacterial DNA kit (Zymo Research, Irvine, USA) according to the manufacturer's

instructions. Briefly, the dry swab material was placed in a sterile 2 ml microcentrifuge tube (Axygen, USA) and 2 ml 1X Phosphate-buffer saline (PBS) (Gibco, Invitrogen, Waltham, USA) buffer was added to re-suspend the bacterial cells. An internal control was added to the samples before DNA extraction commenced to confirm the DNA extraction and rule out PCR inhibition (Choe *et al*., 2013). To a ZR BashingBead Lysis Tube (Zymo Research, Irvine, USA), 100 µl of bacterial cells (that have been re-suspended in PBS) was added and 750 µl Lysis solution. The tube was vortexed (VELP Scientifica, Usmate Velate MB, Italy) for ± 5 min at maximum speed. The ZR BashingBead Lysis Tube (Zymo Research, Irvine, USA) was centrifuged (Spectrafuge, Labnet International, Edison, USA) at ≥10 000 x *g* for 1 min. A volume of 1 200 µl of bacterial DNA buffer was added to the filtrate in the collection tube. A volume of 800 µl of the mixture was transferred to a Zymo-Spin IIC column (Zymo Research, Irvine, USA) in a collection tube and centrifuged (Spectrafuge, Labnet international, Edison, USA) at 10 000 x *g* for 1 min. The flow-through from the collection tube was discarded and the latter step repeated. Two hundred microliters of DNA Pre-Wash Buffer (Zymo Research, Irvine, USA) was added to the Zymo-spin IIC column (Zymo Research, Irvine, USA) in a new collection tube and centrifuged (Spectrafuge, Labnet International, Edison, USA) at 10 000 x *g* for 1 min. A volume of 500 µl bacterial DNA wash buffer was added to the Zymo-spin IIC column (Zymo Research, Irvine, USA) and centrifuged (Spectrafuge, Labnet International, Edison, USA) at 10 000 x g for 1 min. The Zymo-spin IIC column (Zymo Research, Irvine, USA) was transferred to a clean 1.5 ml micro-centrifuge tube (Axygen, Union city, USA) and 100 µl DNA elution buffer (Zymo Research, Irvine, USA) was added directly to the column matrix and centrifuged (Spectrafuge, Labnet International, Edison, USA) at 10 000 x g for 30 s to elute the DNA. The eluted DNA was stored at -20°C until further analysis.

3.3.7 Detection of *C. trachomatis, N. gonorrhoea* and *T. vaginalis* by Xpert CT/NG and Xpert TV

Before the procedure, the GeneXpert (Cepheid, Sunnyvale, USA) instrument was checked to make sure that the six-colour GeneXpert Software Version 4.3 or higher has been uploaded. The test was performed within 30 min of adding the sample to the cartridge. The vaginal swabs were placed inside the tube containing the transport media after collection. In the laboratory 200 µl of Xpert CT/NG Swab sample was mixed with 3 ml of GeneXpert CT/NG swab sample buffer. A volume of 500 µl of this mixture was loaded in the Xpert CT/NG

Assay cartridge and loaded on the GeneXpert automated platform. The results were viewed and printed according to the manufacturer's instructions.

The same procedure was followed for the GeneXpert TV assay. Briefly, a volume of 200 µl of the Xpert TV Swab was mixed with 3 ml of GeneXpert TV Swab sample buffer (Cepheid, Sunnyvale, USA). Five hundred microliter of the mixture was loaded into the Xpert TV Assay cartridge and ran on the GeneXpert automated platform. The *T. vaginalis* run's validity was checked and the results were viewed and printed according to the G*eneXpert Dx System Operator Manual* (Cepheid, Sunnyvale, USA).

3.3.8 Molecular detection of *T. vaginalis* and co-infections by Anyplex II STI-7 Detection PCR

A real-time PCR assay targeting seven microorganisms *(C. trachomatis, M. genitalium, M. hominis, N. gonorrhoeae, T. vaginalis, U. parvum* and *U. urealyticum*) was performed using the Anyplex™ II STI-7 Detection Kit (Seegene, Seoul, Korea), in accordance with the manufacturer's protocol, on a CFX96 real-time thermocycler (Bio-Rad, Munich, Germany). Each PCR reaction mix contained 5 µl of 4x STI-7 TOM, 5 µl of Anyplex PCR Mix, 5 µl of RNase-free water and 5 µl of extracted DNA in a final 20 µl reaction volume. The thermal cycling conditions consisted of an initial incubation at 50°C for 4 min to activate the Uracil-DNA Glycosylase (UDG) system and prevent contamination, pre-denaturation at 95°C for 15 min, followed by 50 cycles of alternating temperatures: 95°C for 30 sec, 60°C for 1 min, and 72°C for 30 sec. The melting temperature was analysed by increasing the reaction temperature from 55°C to 85°C (5 s/ 0.5°C).

3.3.9 Conventional multiplex PCR assay for detection of mycoplasmas and ureaplasmas

A multiplex PCR (M-PCR) assay was performed with primers specific for the highly conserved regions of the multiple-banded antigen gene of *Ureaplasma* spp., the 140-kDa adhesion protein gene of *M. genitalium* and the 16S rRNA gene of *M. hominis* (Stellrecht *et al*., 2004). The nucleotide sequences of the target genes and the expected sizes of the PCR products are displayed in Table 3.2. The Qiagen multiplex PCR kit (Qiagen, Germany) was used and contained 2x Qiagen Multiplex PCR Master Mix, Q-solution and RNase-free water

(Ultrapure quality, PCR-grade, Qiagen, Germany). The multiplex mixture contained the components and their concentrations plus $MgCl_2$ (pH 8.7) at a final concentration of 3 mM. A 10x primer mix of 500 µl was prepared by adding 20 µl of each primer (stock concentration of 50 µM) and TE buffer [10 mM Tris-HC1 (pH 8; Sigma-Aldrich, Germany), 1 mM EDTA (Sigma-Aldrich, Germany)].

The PCR amplification conditions for the conventional M-PCR assay were as follows: Initial denaturation (HotStart*Taq* DNA Polymerase) at 95°C for 15 min; denaturation at 94°C for 30 sec, annealing at 55°C for 1 min and extension at 72°C for 90 sec for 30 cycles and a final extension step at 72°C for 5 min.

All DNA amplicons were analysed using a 2% (m/v) (Metaphor®, Lonza, Switzerland) agarose gel, in 1× Tris-Borate-EDTA (Merck, Germany) buffer (40 mM Tris-HCl, 20 mM borate and 1 mM EDTA, pH 8.5), stained with 5 µL of ethidium bromide (10 $\mu g.mL^{-1}$) (Promega, USA). A 50 base pair (bp) DNA ladder (Fermentas, Thermo Scientific, USA) was included as a molecular weight marker on each gel. The amplicons was visualised under ultraviolet light (Transilluminator, Ultra-violet Products Incorporated, USA) and all visible bands were manually compared relative to the 50 bp DNA ladder (Fermentas, Thermo Scientific, USA).

3.3.10 Data analysis

Statistical analysis of the results was performed using SPSS version 16. Cross-tabulation followed by Chi square (X2) test or Fischer's exact test were used for frequency analysis as appropriate, and a crude odds ratio (COR) with 95% confidence interval (CI) were calculated. Binary regression was applied to measure the odds ratio (OR) and CI of each microorganism and single/ multiple infection on the development of EP. All tests performed were two-sided and P value < 0.05 was considered significant.

Trichomonal infection was the primary outcome of interest. Explanatory variables included demographic characteristics and sexual and drug using behaviours. Categorisation of continuous variables was based on the distribution of data. As odds ratios overestimate the estimated risk when the prevalence is high, we calculated prevalence ratios to avoid that bias. Multivariate models were created taking into account all variables that were significant at *P*-

value <0.05 in univariate analysis. The variables added to the multivariate model were selected based on likelihood ratio tests comparing nested to saturated models and included only if the likelihood ratio test had a *P*-value <0.05.

3.4 Results

A total of 117 samples obtained from female participants attending the public health clinic (35 samples) in Tshwane and a sexual private health clinic (82 samples) in Pretoria East, Silver Lakes, Gauteng were included in this study (Table 1). In the public health clinic the age range was from 22 to 69 years old with an average of 41 and in the private sexual health clinic the age range was from 18 to 56 years old with an average of 34 (Table 3.3)

Bacterial vaginosis (BV) was detected using Nugent scoring system and it showed that 18% (15/82) of the participants had intermediate vaginal flora and 15% (12/82) of the participants were BV positive in the private sexual health clinic. In the public health clinic 53% (18/35) of the participants had intermediate vaginal microflora and no BV positive cases was detected. The InPouch TV culture was performed for 117 specimens for detection of *T. vaginalis* immediately upon receipt and examined for the next five days for the presence of protozoans. The InPouch culture showed the presence of protozoans in 1.7% (2/117) (Table 2). Culture and microscopy for *N. gonorrhoeae* were performed on 117 specimens after collection. Gram stained smears showed Gram-negative intracellular diplococci in 1.7% (2/117) of the participants (Table 3.4).

The Oxidase test, API NH test and GeneXpert PCR assay were performed to confirm *N. gonorrhoeae* and culture positive isolates. The oxidase test and GeneXpert CT/NG confirmed the same two isolates from culture positive isolates to be *N. gonorrhoeae* in contrast with the API NH test which identified *Neisseria* spp. GeneXpert TV confirmed two isolates from InPouch TV positive to be *T. vaginalis* (Table 2).

The Anyplex II STI-7 real-time PCR assay was performed to detect seven STI pathogens (Figure 3.3). From the public health clinic 35 samples were tested using this real-time PCR assay and the results were as follows: Anyplex II STI-7 showed 54% (19/35) for *U. parvum,* 17% (6/35) for *M. hominis* and *U. urealyticum,* 9% (3/35) for *T. vaginalis*, 9% (3/35) for *C. trachomatis* and 3% (1/35) for *N. gonorrhoeae*. In the private sexual health clinic 82

samples were tested using the real-time PCR assay and the results showed 37% (30/82) for *U. parvum,* 11% (9/82) for *T. vaginalis*, 11% (9/82) for *M. hominis,* 5% (4/82) for *U. urealyticum,* 2% (2/82) for *C. trachomatis* and 1% (1/82) for *N. gonorrhoeae*. *Trichomonas vaginalis* occurred once with *N. gonorrhoeae* at the public health clinic and occurred once with *C. trachomatis* at the private sexual health clinic. The overall prevalence for each of the microorganisms detected using the assay for both clinics was: 41.9% (49/117) of *U. parvum*, 12.8% (15/117) of *M. hominis*, 10.3% (12/117) of *T. vaginalis,* 9.4% (11/117) of *U. urealyticum,* 4.3% (5/117) of *C. trachomatis,* 1.7% (2/117) of *N. gonorrhoeae* (Table 2).

Anyplex II STI-7 real-time PCR confirmed one GeneXpert TV positive isolate as well as three GeneXpert CT/NG positive results for two *C. trachomatis* and one *N. gonorrhoeae*. GeneXpert TV did not detect the eight *T. vaginalis* positive isolates that was detected using the Anyplex II STI-7 real-time PCR (Table 2).

3.5 Discussion

Developing countries experience the challenges of increasing rates in the transmission of microorganisms causing asymptomatic STIs (Stoneburner & Low-Beer, 2004). Risk factors that influence the increased rate of STIs amongst populations of developing countries have been identified as a combination of behavioural, social, demographics and economics (Stoneburner & Low-Beer, 2004). The lack of education, alcohol and drug abuse as well as multiple sexual partners has been recognised as significant contributing factors to the increasing rate of trichomoniasis (Flint & du Plessis, 2013). Within South Africa the additional contributing factor behind the increased rate of STIs is a common phenomenon of migration by men from rural to urban areas for employment (Flint & du Plessis, 2013). Worldwide prevalence of *T. vaginalis* varies from 2% to greater than 50% depending on the region, country, gender and demographic parameters of the study population as well as the diagnostic procedures that were used in various studies (Ginococchio *et al.,* 2012).

The prevalence of *T. vaginalis* is not well characterized primarily because *T. vaginalis* infections are not reportable to public health officials and are generally not part of routine screening. Published studies that generally measured *T. vaginalis* prevalence used relatively insensitive methods. In this study we used molecular methods that are more sensitive to culture which was thought to be the gold standard for detecting *T. vaginalis.* The study

included two clinics namely the public health clinic and a private sexual health clinic serving different populations groups.

In the study, a STI (*C. trachomatis, N. gonorrhoeae* and *T. trachomatis*) prevalence of 13.7% (16/117) was observed. This represent a lower rate compared to the study of Guy *et al.* (2014) that was conducted in Australian Aboriginal communities where STI prevalence of 33.3% was reported. A prevalence of 10.3% (3463/10 396) for *T. vaginalis* was observed in this study, which is in agreement with findings reported by De Jongh *et al.* (2010) of 12.5% in women presenting at a termination of pregnancy clinic in Pretoria, South Africa. Naidoo and Wand (2013) reported a lower prevalence of 6.2% for *T. vaginalis* in Durban, South Africa. For *C. trachomatis* an overall prevalence of 4.3% was observed in this study. Compared to this study, De Jongh *et al.* (2010) in a cohort studied in Pretoria, South Africa and Guy *et al* (2014) in Aboriginal communities, Australia reported prevalence rates of 4.5% and 9.4% respectively. Ginococchio *et al* (2012), Guy *et al* (2014) and De Jongh *et al* (2010) reported a very high prevalence of *C. trachomatis* 6.7%, 12.3% and 14.0% respectively. The aforementioned studies are in support of this study that *C. trachomatis* is frequently detected.

A prevalence of 1.7% *N. gonorrhoeae* was observed in this study. A study conducted in the United States (US) by Ginococchio *et al* (2012) reported the same prevalence of 1.7%. When the two clinics sampled in this study are compared, *T. vaginalis* (9%) and *C. trachomatis* (9%) infections were high in the public health clinic where in the private sexual health clinic *T. vaginalis* was detected in 11% and *C. trachomatis* 3% of participants. The population of different races might contribute to the high number of positive cases at the public health clinic. One other reason for the high number of cases might be the high migration rate by partners from rural areas to urban areas in search of employment where they have sexual relationships with other partners.

According to the study conducted by Ginococchio *et al.* (2012) in the United States (US) in women aged 18 to 89 years across 21 states, the rates of *T. vaginalis, C. trachomatis* and *N. gonorrhoeae* co-infections were low (<1.3%) in the whole population. The same study showed that among women in the USA the prevalence of dual infections were much higher in African-Americans women aged 18 to 89 years than Caucasian women: *C. trachomatis/ T. vaginalis* (3.6% vs 0.6%), *C. trachomatis/ N. gonorrhoeae* (1.7% vs 0.4%) and

T. vaginalis/ N. gonorrhoeae (1.7% vs 0.5%). Other studies reported co-infection rates in high-risk populations or reported *C. trachomatis* and *N. gonorrhoeae* prevalence in *T. vaginalis*-positive women (Sutton *et al.,* 2007; Allsworth *et al.,* 2009). In this study the rate of co-infection was 2.6% which is slightly higher than the rate reported in the Ginococchio *et al* (2012) study. Dual infections of 12.5% were observed for *T. vaginalis/ C. trachomatis* and 6.25% for *T. vaginalis/ N. gonorrhoeae.* These findings were supported by the Naidoo and Wand (2013) study in Durban, who reported 17% for *T. vaginalis/ C. trachomatis* and 5% for *T. vaginalis/ N. gonorrhoeae.*

Both co-infections were common in women <30 years old of age in Ginococchio *et al.* (2012), probably because *C. trachomatis* and *N. gonorrhoeae* infections are more prevalent in this age group (Ginococchio *et al.,* 2012). This study supports our study because the dual infections between *T. vaginalis/ C. trachomatis* and *T. vaginalis/ N. gonorrhoeae* occurred in women between the ages of 26 to 32 years of age. *Trichomonas vaginalis* was associated with both *N. gonorrhoeae* and *C. trachomatis* in nearly all age groups of women and Ginococchio *et al.* (2012) reported the same findings. These findings may suggest that there may be a stronger biological link between the factors related to transmission or acquisition of *N. gonorrhoeae* and *T. vaginalis* than those related to *C. trachomatis.* Therefore further research studies are needed to confirm this suspected biological link *in vivo* and *in vitro* which can involve fluorescent *in situ* hybridisation (FISH) assays with confocal microscopy to view the phenotypic interaction of the bacteria and human cells. The use of next generation sequencing (NGS) technology focusing on the transcriptomes may uncover genes upregulated or downregulated when these two bacteria are in close proximity.

Leon *et al* (2009) reported a strong association between trichomonal infection and increased age amongst women in Coastal Peru (Leon *et al* 2009). In this study, age was not significantly associated with prevalent infections; the findings were also supported by the Naidoo and Wand (2013) study. Another study found that women having sex using condom had significantly lower rates of *T. vaginalis* (Kissinger *et al.,* 2009). Significant risk factors for incident *T. vaginalis* infection included having ≥ three life-time sexual partners, infection with any STI at screening and the Zulu language (Naidoo & Wand, 2013). Naidoo and Wand (2013) also found that unmarried non-cohabiting women were at a higher risk of having a trichomonas infection.

This study showed that being unmarried was significantly associated with *T. vaginalis* at the private sexual health clinic and it was also associated with any STI detected (*C. trachomatis, N. gonorrhoeae* and *T. vaginalis*) at both clinics. Condom use and tampon use were found to be associated with *C. trachomatis, N. gonorrhoeae* and *T. vaginalis* at both clinics. In a multivariate analysis of the data obtained from the private sexual health clinic, condom use was the only independent significant factor. Tampon use was the only variable at the public health clinic that remained significant in the multivariate analysis. Research suggests that HIV-positive individuals with an STI may be at increased risk of passing HIV to someone else through anal, vaginal and frontal sex (Johnson and Lewis, 2008). But there was no significant association of STIs and HIV status in this study due to the small sample size.

Mycoplasmas and ureaplasmas were also investigated in this study using only Anyplex II STI-7 PCR (Seegene, Seoul, Korea) and convetional multiplex PCR assay. *Ureaplasma parvum* was the most commonly identified species and contributed to the high prevalence of genital mycoplasmas found in this study with Anyplex II STI-7 PCR. Several studies like Povlsen *et al.* (2001) and Kataoka *et al.* (2006) also reported *U. parvum* to be the most prevalent genital mycoplasma in reproductive-age women with prevalence rates of 17% to 64%. Bayraktar *et al.* (2010) and Redelinghuys *et al.* (2014) reported *M. hominis and U. parvum* of up to 80% which confirms that these genital mycoplasmas are colonisers found in the vaginal milieu. With regards to clinics, the public health clinic showed the highest prevalent of *U. parvum* of 54% (19/35) compared to 37 (30/82) for the private sexual health clinic. Overall, all the genital mycoplasma species, except *M. genitalium* (0%) and *U. urealyticum* (9.4%) were present in high numbers in the samples. Inconsistent findings exist as to which ureaplasma species is the most pathogenic (Kataoka *et al.,* 2006, Govender *et al.,* 2009). However, Andrade-Rocha (2003) reported that *U. urealyticum* is potentially the pathogenic species playing an aetiologic role in both genital infections and male infertility (Andrade-Rocha, 2003). During the past decades, evidence for damage caused by *U. urealyticum* to the development and vitality of human embryos has accumulated (Montagut *et al.,* 1991). In human *in vitro* fertilization studies, the presence of *U. urealyticum* in either semen or the female genital tract resulted in a decline in pregnancy rate per embryo transfer (Reichart *et al.,* 2000). The results found using the conventional multiplex PCR assay were not as easily interpretable compared to the results of the Anyplex II STI-7 PCR assay (Figure 3.4). It was also a time-consuming process despite being a cheaper assay to use to detect the different mycoplasmas.

The prevalence of BV found in the private health clinic (16%) corresponds with the prevalence rates of 5% to 25% in (asymptomatic) women resported by Tolosa *et al.* (2006) conducted in eight institutions participating in the global network for prenatal and reproductive health (Figure 3.1). *Mycoplasma hominis* was present in 3.7% of women with BV and *U. parvum* in 8.5% of women with BV. The association rate between BV and the mycoplasmas was not determined. The potential impact of the prevalent BV and genital mycoplasmas should be investigated in more defined populations focusing on maternal and foetal health.

3.6 Conclusion

The study provides a robust indication of the true burden over time of *T. vaginalis* among participants presenting at two clinics and this indicates that it is of a public health concern. The high *T. vaginalis* prevalence with its co-infections confirms the need for simultaneous screening for *C. trachomatis, N. gonorrhoeae* and *T. vaginalis.* The Anyplex II STI-7 real time PCR assay was a good diagnostic tool with its ease of use and processing, which makes it worthwhile to incorporate into the day to day laboratory work. The GeneXpert assays need to be installed at health care clinics as point-of-care tools as it provided rapid results with minimum hands-on time.

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Table 3.1: Nugent scoring system (0 to 10) for Gram-stained vaginal smears (Nugent *et al.*, 1991)

Score	*Lactobacillus* spp.	*Gardnerella vaginalis* and *Bacteroides* spp. morphotypes	*Mobiluncus* spp.
0	4+	0	0
1	3+	1+	1+/2+
2	2+	2+	3+/4+
3	1+	3+	
4	0	4+	

[1] Morphotypes are scored as the average number seen per oil immersion field. Less weight is given to curved Gram-variable rods. Total score = lactobacilli + *G. vaginalis* and *Bacteroides* spp. + curved rods.

Table 3.2: Nucleotide sequences of primers used in the detection of genital mycoplasmas (Stellrecht *et al.*, 2004)

Organism and Primer	Target	DNA sequence (5'-3')	Expected sizes (bp)
M. genitalium	140-kDa adhesion protein gene	MG1 primer: AGTTGATGAAACCTTAACCCCTTGG MG2 primer: CCGTTGAGGGGTTTTCCATTTTTGC	282 bp
M. hominis	16S rRNA	RNAH1 primer: CAATGGCTAATGCCGGATACGC RNAH2 primer: GGTACCGTCAGTCTGCAAT	334 bp
U. urealyticum and *U. parvum*	MB antigen gene	UMS125 primer: GTATTTGCAATCTTTATATGTTTTCG UMA226 primer: CAGCTGATGTAAGTGCAGCATTAAATTC	403 and 448 bp (respectively)

Table 3.3: Demographics of the patients from the two clinics

Variables	Clinics		P- value
	My Sexual health clinic N=82	Skinner clinic N=35	
Age	33.50 (18-56)	40.00 (22-69)	0.006
Ethnicity (n=113)			0.000
Black	18 (22.5%)	33 (100%)	
Caucasian	60 (75.0%)	0	
Coloured	2 (2.5.0%)	0	
Marital status (n=111)			0.063
Single	31 (39.7%)	21 (46.8%)	
Married	46 (59.0%)	58 (52.3%)	
Divorced	1 (1.3%)	1 (0.9%)	
HIV status (n=111)			0.748
Positive	8 (10.3%)	4 (12.1%)	
Negative	70 (89.7%)	29 (87.9%)	
Number of Pregnancy lost (n=111)			0.808
None	69 (88.5%)	98 (88.3%)	
One	8 (10.3%)	11 (9.9%)	
Two	1 (1.3%)	2 (1.8%)	
New sexual partner (6months) (n=111)			0.603
Yes	16 (20.5%)	5 (15.2%)	
No	62 (79.5%)	28 (84.8%)	
Condom use (n=111)			0.024
Most of the time	60 (77.9%)	19 (55.9%)	
Usually not	17 (22.1%)	15 (44.1%)	
Alcohol use (n=116)			0.003
Never	35 (43.2%)	26 (74.3%)	
Mild	44 (54.3%)	7 (20.0%)	
Extensive	2 (2.5%)	2 (5.7%)	
Tampon use (n=115)			0.000
Yes	50 (61.7%)	3 (8.8%)	
No	31 (38.3%)	31 (91.2%)	
Average sex frequency	2.00 (0-7)	2.00 (0-5)	0.151

Table 3.4: Prevalence of infections found in the two clinics detected by Anyplex II STI-7 PCR and confirmatory tests

Pathogens	Clinic name		Overall n=117
	My Sexual Health clinic N=82	Skinner clinic N=35	
Trichomonas vaginalis			
Positive	9 (11.0%)	3 (8.6%)	12 (10.3%)
Negative	73 (89.0%)	32 (91.4%)	105 (89.7%)
Neisseria gonorrhoeae			
Positive	1 (1.2%)	1 (2.9%)	2 (1.7%)
Negative	81 (98.8%)	34 (97.1%)	115 (98.3%)
Chlamydia trachomatis			
Positive	2 (2.4%)	3 (8.6%)	5 (4.3%)
Negative	80 (97.6%)	32 (91.4%)	112 (95.7%)
Mycoplasma hominis			
Positive	9 (11.0%)	6 (17.1%)	15 (12.8%)
Negative	73 (89.0%)	29 (82.9%)	102 (87.2%)
Mycoplasma genitaluim			
Positive	-	-	-
Negative	82 (0%)	35 (0%)	117 (0%)
Ureaplasma urealyticum			
Positive	5 (6.1%)	6 (17.1%)	11 (9.4%)
Negative	77 (93.9%)	29 (82.9%)	106 (90.6%)
Ureaplasma parvum			
Positive	30 (36.6%)	19 (54.3%)	49 (41.9%)
negative	52 (63.4%)	16 (45.7%)	68 (58.1%)
Any STI detected			
STI detected	10 (12.2%)	6 (17.1%)	16 (13.7%)
No STI detected	72 (87.8%)	29 (82.9%)	101 (86.3%)
Bacterial vaginosis (Nugents scoring system)	57 (69.5%)	16 (47.1%)	73 (62.9%)
Normal	12 (14.6%)	18 (52.9%)	30 (25.9%)
Intermediate	13 (15.9%)	0 (0.0%)	13 (11.2%)
Positive			

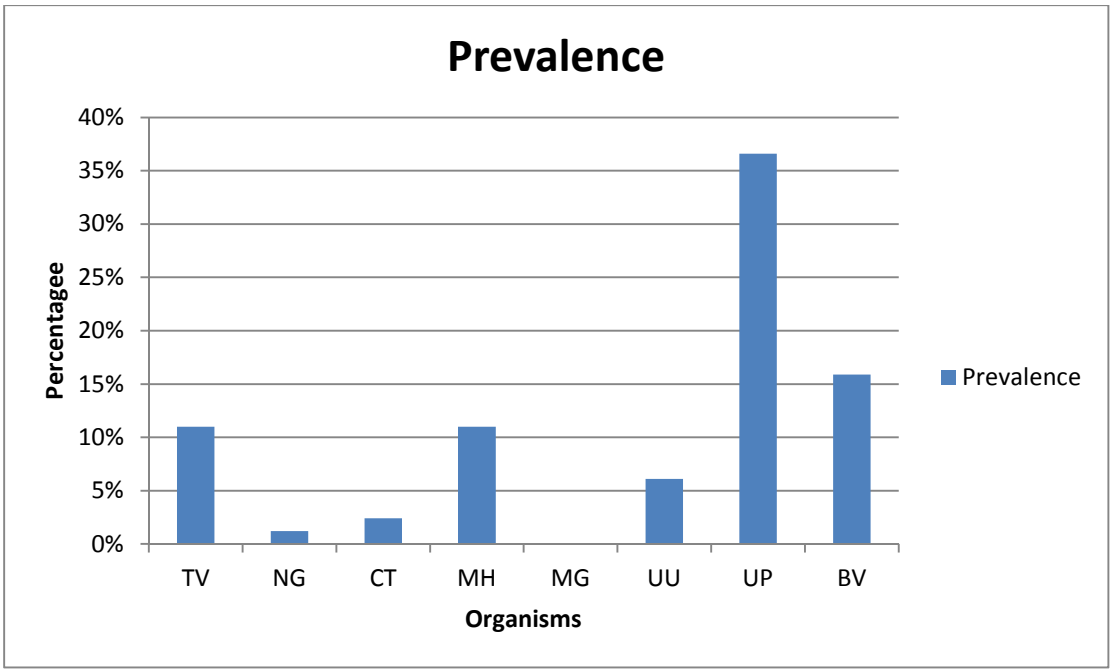

Figure 3.1: Prevalence of infections in the private sexual health clinic in bar chart

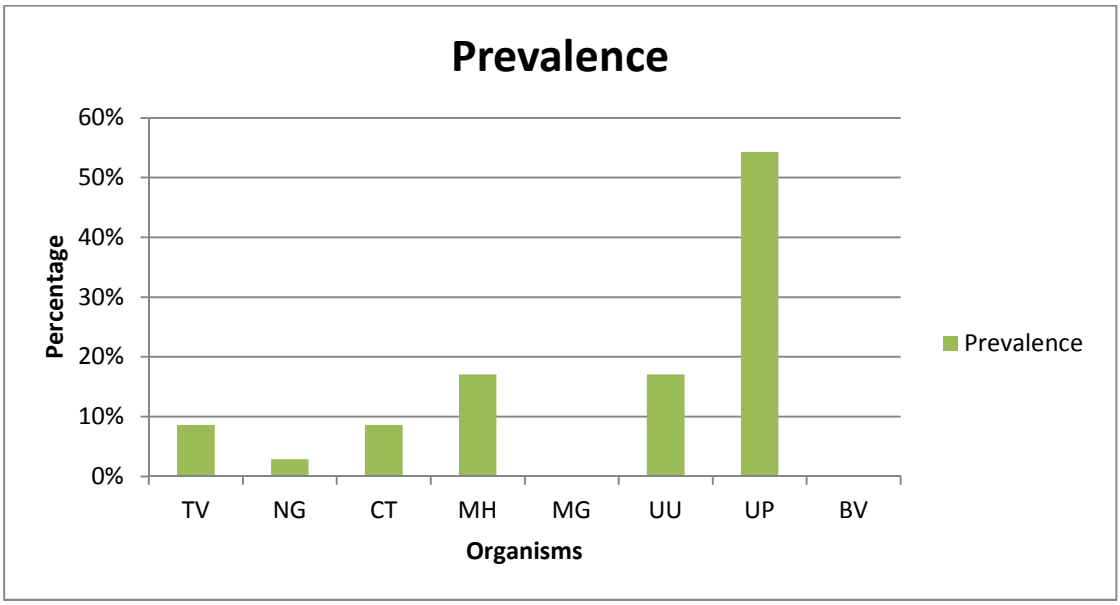

Figure 3.2: Prevalence of infections found in public sexual health clinic in bar chart

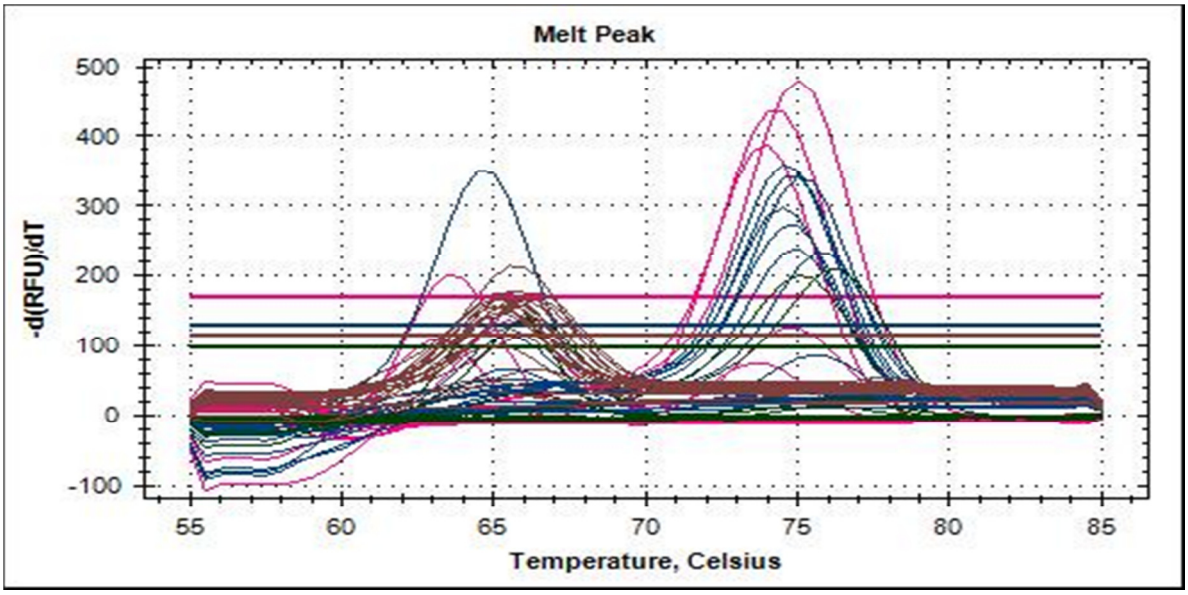

Figure 3.3: Anyplex II STI-7 real-time PCR melting curve analysis for positive and negative specimens

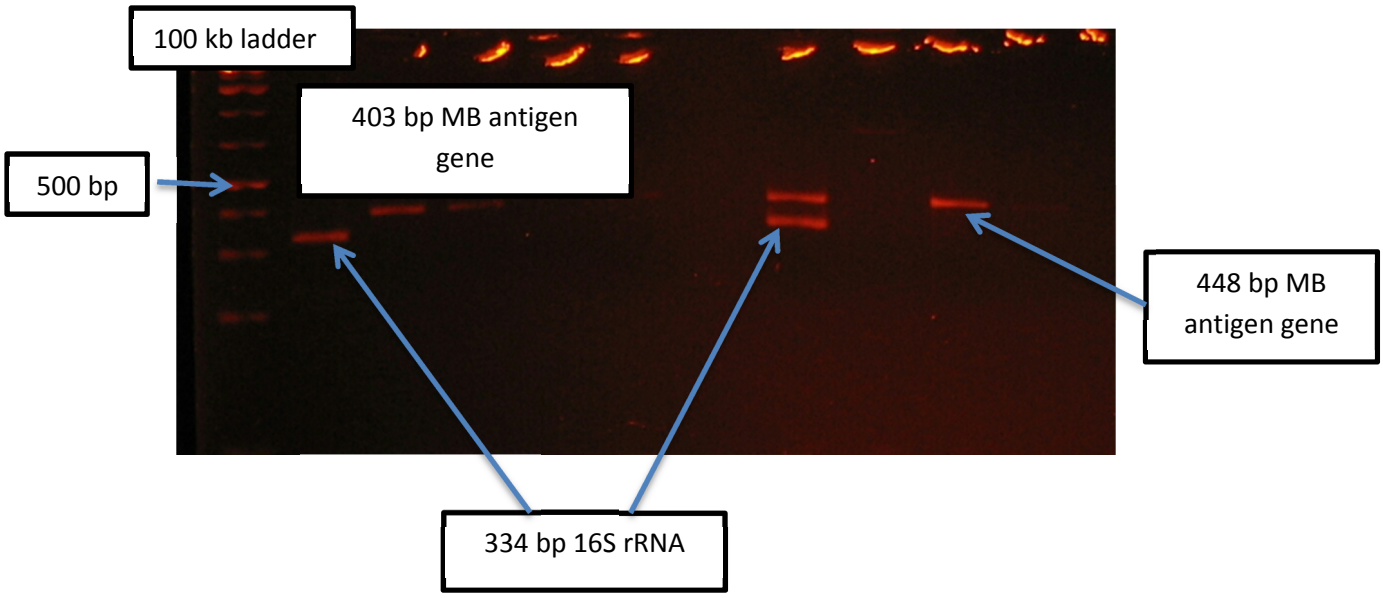

Figure 3.4: Gel electrophoresis picture showing 334 bp 16S rRNA, 403 bp MB antigen gene and 448 bp MB antigen gene detected in *Mycoplasma* and *Ureaplasma* isolates

CHAPTER 4

Concluding remarks

4.1 Conclusions

Sexually transmitted infections (STIs) are major health problems that affect young people (15 to 24 years old) worldwide, especially in developing countries (Visalli *et al.,* 2014). Untreated STIs, particularly gonococcal and chlamydial infection can cause pelvic inflammatory disease (PID) in women and can lead to infertility in both men and women (Muvunyia *et al.,* 2011). Other complications, such as ectopic pregnancy and the risk of developing infections that lead to acute salpingitis may also develop from untreated STIs (Miller, 2006; Muvunyia *et al.,* 2011).

Trichomonads are aerobic flagellated protozoa inhabiting the urogenital and digestive tract of animals and human (Honigberg & Brugerolle, 1990). *Trichomonas vaginalis* is not a reportable STI, unless in the case of HIV-positive women as *T. vaginalis* infection in HIV-infected women might enhance HIV transmission by increasing genital shedding of the virus (Workowski & Berman, 2010). It is most frequently diagnosed by microscopic examination of a wet mount of vaginal fluid which has a sensitivity of 50% to 60% (Krieger *et al.,* 1988; Huppert *et al.,* 2007). The gold standard for the diagnosis of *T. vaginalis* in women has been vaginal fluid culture and is categorised by the Clinical Laboratory Improvement Amendments (CLIA) as moderately complex (Hobbs *et al.,* 2008). However, *T. vaginalis* culture is not readily available in clinical settings and the sensitivity is less (40% to 60%) compared to the available, highly sensitive nucleic acid amplification tests (NAATs) (Nye *et al.,* 2009). The nucleic acid amplification tests run on the same instrumentation platforms as for *C. trachomatis* and *N. gonorroheae* testing (Bachmann *et al.,* 2011; Schwebke *et al.,* 2011). A large proportion of *T. vaginalis* infections are asymptomatic and if left undiagnosed and untreated, *T. vaginalis* increases the risk of perinatal morbidity, such as premature delivery and low birth weight (Muvunyia *et al.,* 2011).

A high prevalence of trichomoniasis among adults in eastern and Southern Africa, ranging from 17% to 42%, has been reported (Watson-Jones *et al.,* 2000; Buvé *et al.,* 2001). High *T. vaginalis* prevalence in all age groups suggests that all women should be considered at risk for STIs in this study. Women (of all ages) with multiple partners or new partners should be

screened for *T. vaginalis* even if they are not screened or tested for *C. trachomatis/ N. gonorrhoeae.* In contrast, Ginococchio *et al* (2012) reported that women ≥50 years old were most likely asymptomatic, as routine *C. trachomatis/ N. gonorrhoeae* screening is not done in this group (Ginococchio *et al.,* 2012).

All the participants included in the study were asymptomatic for STI infections. The high prevalence of *T. vaginalis* infection in these asymptomatic women emphasises the need for *T. vaginalis* to be made a reportable disease by the Centers for Disease Control and Prevention (CDC) because the infection in women is associated with adverse outcomes including pelvic inflammatory disease, concurrent vaginitis and STIs, and it can enhance the risk of HIV (Fichorova, 2009). Point of care testing for the rapid identification and treatment of *T. vaginalis* in asymptomatic populations should therefore be considered by local healthcare providers. The use of a molecular test, particularly Anyplex II STI-7, allowed the comparison of *T. vaginalis, C. trachomatis* and *N. gonorrhoeae* prevalence in various populations, clinical settings and different geographical regions. This study determined the co-infection rate of *T. vaginalis* with *C. trachomatis* and *N. gonorrhoeae* in all asymptomatic women. The inclusion of *C. trachomatis* and *N. gonorrhoeae* as co-infections with *T. vaginalis,* showed 2.6% prevalence as high compared to 1.3% by Ginocchio *et al* (2012). This showed that patients should be screened for *T. vaginalis* even if they are not being tested for *C. trachomatis/ N. gonorrhoeae* (Ginocchio *et al.,* 2012). This confirms the need for increased simultaneous screening for *C. trachomatis, N. gonorrhoeae* and *T. vaginalis* and enhanced strategies to control for these highly prevalent infections.

This study describes the prevalence of and associations between *T. vaginalis* and co-infections as well as genital mycoplasmas in women attending private and public clinics. The high prevalence of genital mycoplasmas found in this study may increase the risk of the women and their unborn babies to develop health problems such as preterm labour leading to preterm birth that can contribute to perinatal mortality (Pararas *et al,*. 2006*)*. This increased risk is a result of potential elevated infections and eventually poor pregnancy outcomes.

A breakdown of the results into the two major clinic groups studied (private and public health care clinics) showed a higher prevalence rate of genital mycoplasma in the participants attending the public health care clinic. Though not statistically significant, this result probably implies that the private health care clinic's population is more aware of their hygiene routine

than the public health care clinic's population. A major difference between the two groups is the socio-economic status of women visiting these two clinics. Another difference is that the the public health care clinic's population belongs mostly to the black race. Black men have more risky sexual behaviour than white men; however, even within different strata of sexual and substance-use behaviour there remain large differences in STI rates between race/ethnicities, particularly for black individuals (Hallfors *et al.,* 2007).

Nucleic acid amplification tests (NAATs) are the most sensitive tests for STI screening and diagnosis (Battle *et al.,* 2001). Nucleic acid amplification tests are more sensitive that the previously available diagnostic tests (e.g. culture, antigen detection and nucleic acid hybridization) by approximately 20% to 30% (Battle *et al.,* 2001). Nucleic acid amplification tests provide an improvement in medical screening for STIs because they can be used with non-invasive specimens, such as first void urine samples and self or clinician-collected vaginal swabs (Gaydos *et al.,* 2013). Multiplex PCR assays have made it convenient for clinicians in many clinical fields to test for multiple causative organisms simultaneously. This is a cost effective diagnostic test because it allows for faster detection and a reduction in labour and reagent cost (Dierkes *et al.,* 2009). Multiplex commercial PCR (Anyplex™ II STI-7 Detection kit) assays were used in this study. This multiplex commercial PCR assay compared to the conventional PCR assay, is cost-saving because it can rapidly detect multiple microorganisms simultaneously through its advanced technology and it is expected that it will be used as a standard diagnostic test for STIs in the near future (Dierkes *et al.,* 2009). The stabilized cost of the kit for use is about R18 000 per 50 sample runs for detecting seven microorganisms.

Detection and treatment of STIs are considered a low-cost opportunity to improve the health of women and is an essential component of HIV control programmes in communities where the burden of STIs is substantial (Hayes *et al.,* 2010). As laboratory based testing is frequently unavailable in low income countries, the World Health Organization (WHO) recommended the syndromic management of STIs for individuals living in such areas (WHO, 2003). Short-falls of the syndromic management approach are that lack of specificity, that there is a risk of overtreatment and that it is unable to detect asymptomatic infections (van der Eem *et al.,* 2016). This has necessitated the need for evaluation of other rapid diagnostic strategies to improve STI management (Romoren *et al.,* 2007).

In the last 15 years, there has been a steady rise in available point-of-care (POC) test for chlamydia and gonorrhoeae diagnosis which produce results in less than an hour based on an antibody/ antigen reaction (Hislop *et al.,* 2010; Huppert *et al.,* 2010). More recently, test systems based on the nucleic acid amplification techniques have been developed that produce results within a few hours (Miners, 2014). Cepheid's GeneXpert CT/NG is a newly approved Food and Drug Administration (FDA) rapid molecular assay for *C. trachomatis* and *N. gonorrhoeae*. The availability of an accurate and portable molecular *T. vaginalis* POC test may also improve *T. vaginalis* screening and management in low-resource settings where there is limited laboratory infrastructure. The evaluation done by Badman *et al.* (2015) suggests that the GeneXpert TV assay is suitable for testing at POC. The GeneXpert TV assay combined with the chlamydia and gonorrhoeae GeneXpert assay would enable health services in remote and low-resource settings to test and treat for all three STIs at the POC using the same GeneXpert platform within a 90 minutes time frame (Badman *et al.,* 2015). This simultaneous testing and detection may improve the time to treatment and reduce the loss to follow up rates which continue to be an issue in these settings. A real-time PCR assay comprises the amplification and fluorescent detection of an amplified DNA target in the same step. Compared with conventional PCR, real-time PCR is cost-effective because it improves the detection sensitivity, decreases the amplification time and simplifies down-stream processing (Edwards *et al.,* 2012). Point of care assays which are affordable, sensitive, specific, user friendly, rapid and robust, equipment free and deliverable are needed in many settings for the accuarate diagnosis of *N. gonorrhoeae* and *C. trachomatics* infections (Peeling *et al.,* 2006). The stabilized cost of the GeneXpert platform for use in STI is about R2 400 per 10 runs for the simultaneous detection of *C. trachomatis* and *N. gonorrhoeae*.

The studied sample size was too small in the two clinic population groups to compare the *C. trachomatis, N. gonorrhoeae* and *T. vaginalis* infection rates between the two clinics. In general, a higher STI infection rate was observed in the participants from the STI public health care clinic compared to participants from the private health care clinic. This observation could be an indication that the private health care clinic group is more health conscious than the public health care clinic group due to proper counseling and testing offered at the private health care clinic. The relatively low rate reported at the private health care clinic could also be due to the treatment given to the participants, thus reducing the rate of STIs; low STI rates might be expected in private health care clinics as participants are routinely screened leading to the treatment of asymptomatic infections. Several limitations

were identified in this study: (i) the samples size was too small to compare the findings between the public and private health care clinics; (ii) no follow-up specimens were taken from the included participants; (iii) STI positive cases received treatment but we did not follow up on the state of the infection.

4.2 Future research

The findings of this study support the continued investigation of rapid and accurate diagnostic tests for the detection of STIs. A larger study to determine the sensitivity and specificity of the latest validated in-house commercially available multiplex real-time PCR assay is required. A rapid point-of-care nucleic acid amplification test (NAAT assay), that can be performed at on-site laboratories or in doctor's room may be an ideal test to guide therapeutic decision making (Caliendo *et al.,* 2013). Gaydos *et al.* (2013) described the Cepheid GeneXpert CT/NG and TV as possible POC tests. The performance of these POC tests should be evaluated in our setting with the high burden of STIs and HIV infection.

The development of next-generation sequencing technologies has greatly enhanced the 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 sequencing technologies in order to enable the very high throughput of sequencing of genomes.

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APPENDIX A

REAGENTS, BUFFERS AND GELS USED IN EXPERIMENTAL PROCEDURES

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

EDTA, disodium salt (Sigma-Aldrich, USA)	93.05 g
Ultrapure water	400 mL
Sodium hydroxide (NaOH) pellets (Merckmillipore, USA)	

Dissolve 93.05 g EDTA in 400 mL ultrapure water, adding the NaOH pellets until the solution becomes clear. Bring the volume to 500 mL and autoclave at 121°C for 15 min

2. Tris-boric EDTA (TBE) buffer, 5X

Tris-base (Sigma-Aldrich, USA)	27 g
Boric acid (Sigma-Aldrich, USA)	13.75 g
0.5 M EDTA (pH 8.0)	10 mL
Ultrapure water	400 mL

Dissolve 27 g of Tris and 13.75 g of Boric acid into 400 mL of ultrapure water. Add 10 mL of EDTA and adjust the pH to 8.0. Bring the volume to 500 mL and autoclave at 121°C for 15 min

3. Brain Heart Infusion (BHI) broth

Brain Heart Infusion broth (LabM Limited, UK)	18.5 g
Ultrapure water	500 mL

Dissolve 18.5 g of BHI broth into 500 mL of ultrapure water. Autoclave at 121°C for 15 min

4. 50% Glycerol solution for long-term storage

Glycerol (Merck Millipore, USA)	250 mL
Ultrapure water	250 mL

Dissolve 250 mL of glycerol into 250 mL of ultrapure water. Autoclave at 121°C for 15 min

5. 2% (m/v) Agarose gels

MetaPhor™ agarose powder (Lonza, USA)	2 g
1X TBE buffer	100 mL
Ethidium bromide [10 mg/mL (Sigma-Aldrich, USA)]	5 µL

Add 1 g of MetaPhor™ agarose powder to 100 mL 1X TBE buffer. Microwave the solution on medium heat for 2 to 3 min, stopping to swirl the solution at intervals. Allow to cool down to 50°C and add 5 µL ethidium bromide. Pour into clean casting tray, add comb and allow to solidify for 30 min.

APPENDIX B

EXPERIMENTAL PROCEDURES

1. Deoxyribonucleic acid (DNA) extraction

1. Add 200 µL of bacterial suspension from the Eswab to the ZR BashingBead™ (Zymo Research, USA) Lysis tube.
2. Add 750 µL of lysis solution and vortex (Disruptor Genie, Scientific Inductries, Inc., USA) at maximum speed for 5 min
3. Centrifuge at 10 000 *g* (Spectrafuge™ 24D, Labnet International Inc, USA) for 1 min
4. Add 400 µL of supernatant to Zymo-Spin™ IV Spin Filter (orange top) (Zymo Research, USA) tube (snap off the base of tube prior to use) in a collection tube and centrifuge at 10 000 *g* (Spectrafuge™ 24D, Labnet International Inc, USA) for 1 min
5. Add 1 200 µL of Fungal/Bacterial DNA binding buffer [with 0.5% 2-mercaptoethanol Merck Millipore, USA)] to the supernatant
6. Transfer 800 µL of the supernatant to Zymo-Spin™ IIC Spin Column (Zymo Research, USA) in a collection tube and centrifuge at 10 000 *g* (Spectrafuge™ 24D, Labnet International Inc, USA) for 1 min. Discard the flow. Repeat step again
7. Add 200 µL of DNA Pre-wash Buffer to Zymo-SpinT^{M} IIC Spin column (Zymo Research, USA) in a new collection tube and centrifuge at 10 000 *g* (Spectrafuge™ 24D, Labnet International Inc, USA) for 1 min
8. Add 500 µL of Fungal/Bacterial DNA wash buffer to Zymo-Spin™ IIC Spin column (Zymo Research, USA) and centrifuge at 10 000 *g* for 1 min
9. Transfer the Zymo-Spin™ IIC Spin column (Zymo Research, USA) to a clean 2 mL microcentrifuge tube (Axygen, Corning, USA) and add 100 µL of DNA elution buffer
10. Centrifuge at 10 000 *g* (Spectrafuge™ 24D, Labnet International Inc, USA) for 30 sec to elute DNA

2. Microscopy of Gram-stained vaginal smears

1. Fix the smear to slides by heating for 5 s using a Bunsen burner, Gram-stained and used for BV diagnosis by the Nugent scoring system
2. Performe Gram staining by flooding the heat-fixed smear with crystal violet (Diagnostic Media Products, NHLS, South Africa) for 1 min
3. Remove excess crystal violet (Diagnostic Media Products, NHLS, South Africa) and add Iodine solution (Diagnostic Media Products, NHLS, South Africa) for 1 min to help bind the first dye to the bacterial cell wall components
4. Decolourise the smear with ethanol (Merck, Germany) for ± 30 s.
5. Counterstain fixed bacterial cells with safranin (Diagnostic Media Products, NHLS, South Africa) for 1 min with the end result of Gram-positive cells appearing purple and Gram-negative cells pink to red under microscopic examination

3. Confirmation of *Neisseria gonorrhoeae* using biochemical tests

1. Add a drop of the substrate tetramethyl-p-phenylene dihydrochloride on the filter paper (BACTIDROP Oxidase, Remel Inc, Lenexa, USA)
2. Moisten the pater with sterile water
3. Pick the colony to be tested using a platinum loop from the culture media and smeared on the filter paper
4. Record the positive results if the filter paper turns blue or purple within 10 to 30 sec
5. Identify positive oxidase isolates using API NH strips (bioMérieux, France) according to the manufacturer's instructions
6. Remove the API NH strip from its individual packaging and place in an incubation box
7. Record the specimen number on the flat portion of the tray
8. Open an ampule of NaCl 0.85% medium with the ampule protector
9. Pick well-isolated colonies using a sterile swab to prepare a suspension with a turbidity equivalent to 4 McFarland, ensuring that it is well mixed
10. Use the suspension immediately after preparation
11. Distribute the prepared bacterial suspension of 50 µl into the first part of seven wells (PEN to URE)
12. Fill the last three wells up to 150 µl
13. Cover the first seven wells (PEN to URE) with mineral oil then incubate for 2 h at 35 ^{o}C to 37^{o}C in aerobic conditions

14. Read the reactions after the incubation period by referring to the package insert included in the kit

15. Store all gonococcal isolates cultured in 50% glycerol in -80°C and -20°C freezers as an integral part of the routine diagnostics

APPENDIX C

CLINICAL DATA OF PATIENTS AND RESULTS FOR DIAGNOSTICS TESTS PERFORMED

Table 1: Patients demographics

Clinic	Lab Number	Date of consent	Age	Ethnicity	Marital status	HIV status	History of pregnancy	New Partner (6months)	Vaginal sex frequently
Skinner	N1	7/28/2015	54	Black	Single	Negative	N/A	No	0
Skinner	O5	9/8/2015	46	Black	Married	Negative	N/A	No	3
Skinner	O7	9/8/2015	55	Black	Single	Negative	N/A	No	2
Skinner	O12	9/8/2015	47	Black	Single	Negative	0	Yes	3
Skinner	O2	9/9/2015	26	Black	Single	Positive	0	No	2
Skinner	O3	9/9/2015	64	Black	Married	Negative	N/A	No	0
Skinner	O6	10/20/2015	40	Black	Married	Positive	0	No	3
Skinner	O8	10/20/2015	28	Black	Single	Negative	N/A	Yes	3
Skinner	O9	10/20/2015	22	Black	Single	Negative	N/A	Yes	2
Skinner	O11	10/22/2015	33	Black	Single	Negative	N/A	No	3
Skinner	O10	10/22/2015	69	Black	Married	Negative	N/A	No	0
Skinner	O1	10/22/2015	61	Black	Single	Negative	N/A	No	0
Skinner	O4	10/22/2015	53	Black	Single	Positive	N/A	No	0
Skinner	D2	11/10/2015	40	Black	Married	Positive	Miscarriage	No	2
Skinner	D4	11/10/2015	23	Black	Single	Negative	N/A	No	3
Skinner	D1	11/10/2015	23	Black	Single	Negative	N/A	No	4
Skinner	D5	11/10/2015	43	Black	Single	Negative	N/A	No	2
Skinner	D3	11/10/2015	54	Black	Single	Negative	N/A	No	0
Skinner	J4	1/26/2016	50	Black	Married	Negative	N/A	No	0

Table 1: Patients demographics (continued)

Clinic	Lab Number	Date of consent	Age	Ethnicity	Marital status	HIV status	History of pregnancy	New Partner (6 months)	Vaginal sex frequently
Skinner	J7	1/26/2016	28	Black	Single	Negative	N/A	No	3
Skinner	J1	1/26/2016	40	Black	Married	Negative	N/A	No	5
Skinner	D6	11/30/2015	25	Black	Single	Negative	N/A	No	4
Skinner	J8	1/27/2016	41	Black	Married	Negative	N/A	No	3
Skinner	J2	1/27/2016	37	Black	Married	Negative	N/A	No	2
Skinner	J5	1/27/2016	65	Black	Single	Negative	Miscarriage	No	0
Skinner	J6	1/27/2016	43	Black	Married	Negative	Miscarriage and normal death	No	3
Skinner	J9	2/2/2016	46	Black	Single	Negative	N/A	No	2
Skinner	J3	2/2/2016	33	Black	Single	Negative	N/A	No	3
Skinner	N6	7/22/2015	45	Black	Single	Negative	N/A	No	2
Skinner	N2	7/28/2015	30	Black	Married	Negative	N/A	No	4
Skinner	N5	7/14/2015	29	Black	Single	Negative	N/A	Yes	3
Skinner	N4	7/14/2015	36	Black	Single	Positive	N/A	Yes	4
Skinner	N7	7/14/2015	35	Black	Single	Negative	N/A	No	3
Skinner	N8	7/13/2015	45	Black	Married	Negative	N/A	No	2
Skinner	N3	8/12/2015	40	Black	Married	Negative	N/A	No	2
MSH clinic	ER1	4/19/2016	39	Black	Single	Positive	None	Yes	1
MSH clinic	ER2	4/19/2016	47	White	Married	Negative	None	No	3
MSH clinic	ER3	4/19/2016	26	White	Married	Negative	None	No	2
MSH clinic	ER4	4/19/2016	21	White	Married	Negative	None	No	0
MSH clinic	ER5	4/22/2016	24	White	Married	Negative	None	Yes	3

Table 3: Patients demographics (continued)

Clinic	Lab Number	Date of consent	Age	Ethnicity	Marital status	HIV status	History of pregnancy	New Partner (6 months)	Vaginal sex frequently
MSH clinic	ER6	4/22/2016	35	White	Married	Negative	Miscarriage at 7 weeks, premature labour on 34 weeks, full term C-section on 37 weeks	No	1
MSH clinic	ER7	4/22/2016	43	White	Married	Negative	Emergency C-section because of slow dilation	No	4
MSH clinic	ER9	4/22/2016	45	White	Married	Negative	None	No	1
MSH clinic	ER10	4/25/2016	27	White	Single	Negative	None	No	4
MSH clinic	ER11	5/10/2016	37	White	Single	Positive	None	No	1
MSH clinic	ER13	5/10/2016	33	White	Married	Negative	ectopic pregnancy, twins at 37 weeks	No	0
MSH clinic	ER14	5/10/2016	18	White	Single	Negative	None	No	1
MSH clinic	ER15	5/10/2016	56	White	Married	Negative	Had trouble falling pregance in both pregnancies	No	3
MSH clinic	ER16	5/9/2016	29	White	Married	Positive	None	No	3
MSH clinic	ER17	6/30/2016	26	White	Married	Negative	None	No	3
MSH clinic	ER17(2)	6/14/2016	26	Black	Single	Negative	None	No	2
MSH clinic	ER18	5/27/2016	29	White	Married	Negative	None	No	3
MSH clinic	ER18(2)	6/14/2016	35	White	Married	Negative	None	No	2
MSH clinic	ER19	6/21/2016	30	White	Married	Negative	None	No	0
MSH clinic	ER20	7/4/2016	48	White	Married	Negative	None	No	3
MSH clinic	ER21	7/26/2016	20	White	Single	Negative	None	No	2
MSH clinic	ER23	7/26/2016	32	Black	Married	Negative	Ectopic	No	2

Table 3: Patients demographics (continued)

Clinic	Lab Number	Date of consent	Age	Ethnicity	Marital status	HIV status	History of pregnancy	New Partner (6 months)	Vaginal sex frequently
MSH clinic	JR1	4/20/2016	35	White	Married	Negative	None	Yes	1
MSH clinic	JR2	4/20/2016	34	White	Married	Negative	Pregnancy loss at 4 weeks	No	2
MSH clinic	JR3	4/20/2016	43	White	Single	Negative	None	No	1
MSH clinic	JR4	4/21/2016	40	White	Married	Negative	None	No	1
MSH clinic	JR5	4/21/2016	30	White	Married	Negative	None	No	3
MSH clinic	JR6	4/27/2016	25	White	Single	Negative	None	No	2
MSH clinic	JR7	4/21/2016	27	White	Single	Negative	None	No	0
MSH clinic	JR9	4/25/2016	37	Coloured	Married	Negative	Miscarriage	No	0
MSH clinic	JR11	4/26/2016	30	White	Married	Negative	none	No	1
MSH clinic	JR12	6/17/2016	28	White	Married	Negative	none	No	2
MSH clinic	JR13	5/17/2016	28	White	Single	Negative	none	Yes	4
MSH clinic	JR15	5/5/2016	48	White	Single	Negative	none	No	0
MSH clinic	JR16	5/11/2016	35	White	Married	Negative	none	No	0
MSH clinic	JR17	5/11/2016	42	White	Married	Negative		No	3
MSH clinic	JR18	5/12/2016	30	Coloured	Single	Negative	none	No	2
MSH clinic	JR19	5/12/2016	45	Black	Single	Negative	none	Yes	0
MSH clinic	JR21	6/10/2016	44	White	Married	Negative	none	No	2
MSH clinic	JR22	6/13/2016	32	White	Married	Negative	none	No	0
MSH clinic	JR23	6/15/2016	43	White	Married	Negative		No	2
MSH clinic	JR24	6/15/2016	27	White	Single	Negative	none	No	2
MSH clinic	JR25	6/20/2016	30	White	Single	Negative	none	No	3
MSH clinic	JR27	7/1/2016	35	Black	Single	Negative	none	Yes	

Table 3: Patients demographics (continued)

Clinic	Lab Number	Date of consent	Age	Ethnicity	Marital status	HIV status	History of pregnancy	New Partner (6 months)	Vaginal sex frequently
MSH clinic	JR29	7/21/2016	30	White	Married	Negative	none	No	1
MSH clinic	JR26	8/30/2016	27	Black	Single	Positive	none	No	2
MSH clinic	JR28	7/29/2016	35	Black	Single	Negative	none	Yes	2
MSH clinic	JR30	7/28/2016	24	White	Single	Negative	none	No	0
MSH clinic	JR31	8/1/2016	30	White	Married	Negative	none	No	3
MSH clinic	JR32	8/4/2016	41	White	Married	Negative	none	No	2
MSH clinic	JR33	8/4/2016	30	White	Married	Negative	none	No	2
MSH clinic	JR34	8/18/2016	28	White	Married	Negative	none	No	3
MSH clinic	JR35	8/22/2016	39	White	Married	Negative	none	No	3
MSH clinic	JR36	8/29/2016	31	White	Married	Negative	none	No	3
MSH clinic	JR37	8/30/2016	48	White	Married	Negative	C-section both pregnancy	No	3
MSH clinic	JS1	4/21/2016	47	White	Married	Negative	none	No	0
MSH clinic	JS2	4/21/2016	35	Black	Married	Negative	none	No	1
MSH clinic	JS3	4/21/2016	28	White	Single	Negative	none	Yes	1
MSH clinic	JS4	4/22/2016	29	White	Single	Negative	none	No	1
MSH clinic	JS5	4/25/2016	21	White	Single	Negative	none	No	4
MSH clinic	JS6	4/25/2016	30	Black	Married	Positive	N/A	No	0
MSH clinic	JS6(2)	5/4/2016	32	White	Married	Negative	none	No	3
MSH clinic	JS7	5/5/2016	30	White	Single	Negative	none	Yes	2
MSH clinic	JS8	5/5/2016	41	White	Married	Negative	N/A	No	2
MSH clinic	JS9	5/23/2016	51	White	Married	Negative	N/A	No	3
MSH clinic	JS10	5/24/2016	34	White	Married	Negative	N/A	No	0
MSH clinic	JS11	5/27/2016	45	Black	Single	Positive	N/A	No	4

Table 3: Patients demographics (continued)

Clinic	Lab Number	Date of consent	Age	Ethnicity	Marital status	HIV status	History of pregnancy	New Partner (6 months)	Vaginal sex frequently
MSH clinic	JS12	5/27/2016	39	Black	Married	Negative	N/A	Yes	2
MSH clinic	JS13	6/10/2016	45	Black	Divorced	Positive	N/A	Yes	3
MSH clinic	JS14	6/15/2016	36	White	Married	Negative	N/A	Yes	2
MSH clinic	JS15	7/6/2016	34	White	Single	Negative	N/A	Yes	1
MSH clinic	JS16	7/7/2016	44	Black	Single	Positive	N/A	Yes	0
MSH clinic	JS19	7/21/2016	39	White	Single	Negative	N/A	Yes	7
MSH clinic	JS21	7/25/2016	48	White	Single	Negative	N/A	No	2
MSH clinic	JS20	7/22/2016	26	White	Single	Negative	N/A	No	2
MSH clinic	JA1	4/22/2016	39	Black	Married	Negative	Ectopic pregancy	Yes	2
MSH clinic	JA2	5/13/2016	38	White	Single	Negative	None	No	4
MSH clinic	JA3	5/24/2016	25	White	Single	Negative	N/A	Yes	2
MSH clinic	JA4	6/27/2016	32	White	Single	Negative	N/A	No	2
MSH clinic	JA5	8/10/2016	21	White	Single	Negative	N/A	No	1
MSH clinic	E8	4/22/2016	29	White	Married	Negative	N/A	No	2
MSH clinic	AD	4/30/2016	36	Black	Married	Negative	N/A	No	2

MSH- My sexual health, N/A- Not available

Table 2: Results for diagnostics tests performed

Lab Number	InPouch TV	GeneXpert TV	GeneXert CT/NG	Oxidase test	*Trichomonas Vaginalis*	*Neisseria gonorrhoeae*	*Chlamydia trachomatis*	*Mycoplasma hominis*	*Mycoplasma genitalium*	*Ureaplasma urealyticum*	*Ureaplasma pervum*
N1	Negative	Negative	Negative	Negative	No	No	No	Yes	No	No	No
O5	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No
O7	Positive	Negative	Negative	Negative	Yes	No	No	Yes	No	No	No
O12	Negative	Negative	Negative	Negative	No	No	No	No	No	No	Yes
O2	Positive	Positive	NG	Positive	Yes	Yes	No	Yes	No	No	Yes
O3	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No
O6	Negative	Negative	Negative	Negative	No	No	No	Yes	No	No	Yes
O8	Negative	Negative	Negative	Negative	No	No	No	No	No	No	Yes
O9	Negative	Negative	Negative	Negative	No	No	No	No	No	Yes	Yes
O11	Negative	Negative	Negative	Negative	No	No	No	No	No	No	Yes
O10	Negative	Negative	Negative	Negative	No	No	No	No	No	No	Yes
O1	Negative	Negative	Negative	Negative	No	No	No	No	No	No	Yes
O4	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No

Table 2: Results for diagnostics tests performed (continued)

Lab Number	InPouch TV	GeneXpert TV	GeneXert CT/NG	Oxidase test	*Trichomonas Vaginalis*	*Neisseria gonorrhoeae*	*Chlamydia trachomatis*	*Mycoplasma hominis*	*Mycoplasma genitalium*	*Ureaplasma urealyticum*	*Ureaplasma pervum*
D2	Negative	Negative	Negative	Negative	No	No	No	No	No	No	Yes
D4	Negative	Negative	Negative	Negative	No	No	No	No	No	Yes	Yes
D1	Negative	Negative	Negative	Negative	Yes	No	No	Yes	No	No	Yes
D5	Negative	Negative	Negative	Negative	No	No	No	No	No	No	Yes
D3	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No
J4	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No
J7	Negative	Negative	Negative	Negative	No	No	No	No	No	Yes	No
J1	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No
D6	Negative	Negative	CT	Negative	No	No	Yes	No	No	No	Yes
J8	Negative	Negative	Negative	Negative	No	No	No	No	No	No	Yes
J2	Negative	Negative	Negative	Negative	No	No	No	No	No	Yes	Yes
J5	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No
J6	Negative	Negative	Negative	Negative	No	No	No	Yes	No	Yes	No
J9	Negative	Negative	Negative	Negative	No	No	No	No	No	No	Yes
J3	Negative	Negative	Negative	Negative	No	No	No	No	No	Yes	No
N6	Negative	Negative	Negative	Negative	No	No	Yes	No	No	No	No
N2	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No
N5	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No
N4	Negative	Negative	CT	Negative	No	No	Yes	No	No	No	Yes
N7	Negative	Negative	Negative	Negative	No	No	No	No	No	No	Yes
N8	Negative	Negative	Negative	Negative	No	No	No	No	No	No	Yes
N3	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No

Table 2: Results for diagnostics tests performed (continued)

Lab Number	InPouch TV	GeneXpert TV	GeneXert CT/NG	Oxidase test	*Trichomonas Vaginalis*	*Neisseria gonorrhoeae*	*Chlamydia trachomatis*	*Mycoplasma hominis*	*Mycoplasma genitalium*	*Ureaplasma urealyticum*	*Ureaplasma pervum*
ER1	Negative	Negative	Negative	Negative	No	No	No	No	No	No	Yes
ER2	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No
ER3	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No
ER4	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No
ER5	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No
ER6	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No
ER7	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No
ER9	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No
ER10	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No
ER11	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No
ER13	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No
ER14	Negative	Negative	Negative	Negative	No	No	No	No	No	No	Yes
ER15	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No
ER16	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No
ER17	Negative	N/T	N/T	Negative	No	No	No	No	No	No	Yes
ER17(2)	Negative	N/T	N/T	Positive	No	Yes	No	Yes	No	No	Yes
ER18	Negative	N/T	N/T	Negative	No	No	No	No	No	Yes	Yes
ER18(2)	Negative	N/T	N/T	Negative	No	No	No	No	No	No	No
ER19	Negative	N/T	N/T	Negative	Yes	No	No	No	No	No	No
ER20	Negative	N/T	N/T	Negative	No	No	No	No	No	No	Yes
ER21	Negative	N/T	N/T	Negative	No	No	No	No	No	No	Yes

Table 2: Results for diagnostics tests performed (continued)

Lab Number	InPouch TV	GeneXpert TV	GeneXert CT/NG	Oxidase test	*Trichomonas Vaginalis*	*Neisseria gonorrhoeae*	*Chlamydia trachomatis*	*Mycoplasma hominis*	*Mycoplasma genitalium*	*Ureaplasma urealyticum*	*Ureaplasma pervum*
ER23	Negative	N/T	N/T	Negative	No	No	No	No	No	No	No
JR1	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No
JR2	Negative	Negative	Negative	Negative	No	No	No	Yes	No	No	Yes
JR3	Negative	Negative	Negative	Negative	No	No	No	No	No	No	Yes
JR4	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No
JR5	Negative	Negative	Negative	Negative	No	No	No	No	No	No	Yes
JR6	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No
JR7	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No
JR9	Negative	Negative	Negative	Negative	No	No	No	No	No	Yes	No
JR11	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No
JR12	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No
JR13	Negative	N/T	N/T	Negative	No	No	No	No	No	No	No
JR15	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No
JR16	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No
JR17	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No
JR18	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No
JR19	Negative	Negative	Negative	Negative	No	No	No	No	No	No	Yes
JR21	Negative	N/T	N/T	Negative	No	No	No	No	No	No	No
JR22	Negative	N/T	N/T	Negative	No	No	No	No	No	No	No
JR23	Negative	N/T	N/T	Negative	No	No	No	No	No	No	No
JR24	Negative	N/T	N/T	Negative	Yes	No	Yes	No	No	No	No

Table 2: Results for diagnostics tests performed (continued)

Lab Number	InPouch TV	GeneXpert TV	GeneXert CT/NG	Oxidase test	*Trichomonas Vaginalis*	*Neisseria gonorrhoeae*	*Chlamydia trachomatis*	*Mycoplasma hominis*	*Mycoplasma genitalium*	*Ureaplasma urealyticum*	*Ureaplasma pervum*
JR25	Negative	N/T	N/T	Negative	Yes	No	No	No	No	No	No
JR27	Negative	N/T	N/T	Negative	Yes	No	No	No	No	No	No
JR29	Negative	N/T	N/T	Negative	No	No	No	No	No	No	No
JR26	Negative	N/T	N/T	Negative	No	No	No	No	No	No	No
JR28	Negative	N/T	N/T	Negative	No	No	No	Yes	No	No	Yes
JR30	Negative	N/T	N/T	Negative	No	No	No	No	No	No	Yes
JR31	Negative	N/T	N/T	Negative	No	No	No	No	No	No	Yes
JR32	Negative	N/T	N/T	Negative	No	No	No	No	No	No	No
JR33	Negative	N/T	N/T	Negative	No	No	No	No	No	No	No
JR34	Negative	N/T	N/T	Negative	Yes	No	No	No	No	No	No
JR35	Negative	N/T	N/T	Negative	No	No	No	Yes	No	No	Yes
JR36	Negative	N/T	N/T	Negative	No	No	No	No	No	No	No
JR37	Negative	N/T	N/T	Negative	No	No	No	No	No	No	No
JS1	Negative	Negative	N/T	Negative	No	No	No	No	No	No	No
JS2	Negative	Negative	Negative	Negative	No	No	No	No	No	No	Yes
JS3	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No
JS4	Negative	Negative	Negative	Negative	No	No	No	Yes	No	Yes	No
JS5	Negative	Negative	Negative	Negative	No	No	No	No	No	No	Yes
JS6	Negative	Negative	Negative	Negative	No	No	No	No	No	No	Yes
JS6(2)	Negative	Negative	Negative	Negative	No	No	No	No	No	No	Yes
JS7	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No
JS8	Negative	Negative	Negative	Negative	No	No	No	No	No	No	Yes

Table 2: Results for diagnostics tests performed (continued)

Lab Number	InPouch TV	GeneXpert TV	GeneXert CT/NG	Oxidase test	*Trichomonas Vaginalis*	*Neisseria gonorrhoeae*	*Chlamydia trachomatis*	*Mycoplasma hominis*	*Mycoplasma genitalium*	*Ureaplasma urealyticum*	*Ureaplasma pervum*
JS9	Negative	N/T	N/T	Negative	No	No	No	Yes	No	No	No
JS10	Negative	N/T	N/T	Negative	No	No	No	Yes	No	No	No
JS11	Negative	N/T	N/T	Negative	No	No	No	No	No	No	Yes
JS12	Negative	N/T	N/T	Negative	No	No	No	No	No	Yes	Yes
JS13	Negative	N/T	N/T	Negative	Yes	No	No	No	No	No	Yes
JS14	Negative	N/T	N/T	Negative	No	No	No	No	No	Yes	Yes
JS15	Negative	N/T	N/T	Negative	Yes	No	No	No	No	No	No
JS16	Negative	N/T	N/T	Negative	Yes	No	No	No	No	No	Yes
JS19	Negative	N/T	N/T	Negative	No	No	No	No	No	No	Yes
JS21	Negative	N/T	N/T	Negative	No	No	No	No	No	No	Yes
JS20	Negative	N/T	N/T	Negative	No	No	No	Yes	No	No	Yes
JA1	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No
JA2	Negative	Negative	Negative	Negative	No	No	No	Yes	No	No	Yes
JA3	Negative	N/T	N/T	Negative	No	No	No	No	No	No	No
JA4	Negative	N/T	N/T	Negative	Yes	No	Yes	No	No	No	No
JA5	Negative	N/T	N/T	Negative	No	No	No	No	No	No	Yes
E8	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No
AD	Negative	Negative	Negative	Negative	No	No	No	No	No	No	No

CT- *Chlamydia trachomatis,* NG- *Neisseria gonorrhoeae,* NT- Not tested, TV- *Trichomonas vaginalis*

APPENDIX D

RESEARCH ETHICS APPROVAL LETTER

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 20 Oct 2016.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 22/04/2017.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

30/04/2015

Approval Certificate
New Application

Ethics Reference No.: 103/2015

Title: Trichomonas vaginalis and bacterial co-infections identified in reproductive age women

Dear Mr Jonas Sethowa

This **New Application** as supported by documents specified in your cover letter dated 24/03/2015 for your research received on the 26/03/2015, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 29/04/2015.

Please note the following about your ethics approval:
- Ethics Approval is valid for 2 years
- Please remember to use your protocol number (103/2015) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

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

Additional Conditions:
- Approved, on condition that permissions be obtained at the research sites and submitted to our REC.

We wish you the best with your research.

Yours sincerely

Dr R Sommeraj MB;ChB; Mmed (Int); MPharMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

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