Detection and characterisation of mycoplasmas in women visiting an antenatal or reproductive biology clinic

by

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I declare that the dissertation, which I hereby submit for the degree MSc (Medical Microbiology) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE: .... DATE: ....

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LIST OF SYMBOLS AND ABBREVIATIONS

SYMBOLS

ºC	Degree Celsius
µL	Microlitre
mL	Milliliter

ABBREVIATIONS

ATP	Adenosine triphosphate
AF	Amniotic fluid
BV	Bacterial vaginosis
C. trachomatis	*Chlamydia trachomatis*
CLD	Chronic lung diseases
CDSs	Specific coding sequences
CDSS	Clinical decision support system
DNA	Deoxyribonucleic acid
EMP	Embden-Meyerhoff-Parnas
HIV	Human immunodeficiency virus
Kb	Kilobase
KOH	Potassium hydroxide
$MgCl_2$	Magnesium chloride
M. genitalium	*Mycoplasma genitalium*
MgPa	*Mycoplasma genitalium* adhesin protein
M. hominis	*Mycoplasma hominis*
NHLS	National Health Laboratory Service
NHANES	National Health and Nutrition Examination Survey
N. gonorrhoeae	*Neisseria gonorrhoeae*
NGU	Non-gonococcal urethritis
PDA	Patent ductus arteriosus

PID	Pelvic inflammatory disease
PBS	Phosphate buffered saline
PTS	Phosphotransferase transport system
PCR	Polymerase chain reaction
PTL	Preterm labour
PPROM	Preterm premature rupture of membranes
RDS	Respiratory distress syndrome
rRNA	Ribosomal ribonucleic acid
STDs	Sexually transmitted diseases
STis	Sexually transmitted infections
T. vaginalis	*Trichomonas vaginalis*
UV	Ultra violet
UK	United Kingdom
US	United States
UDG	Uracil- DNA glycosylase
U. parvum	*Ureaplasma parvum*
U. urealyticum	*Ureaplasma urealyticum*
WHO	World Health Organization

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DETECTION AND CHARACTERISATION OF MYCOPLASMAS IN WOMEN VISITING AN ANTENATAL OR REPRODUCTIVE BIOLOGY CLINIC

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SUMMARY

Genital mycoplasmas and other co-infections are very prevalent causes of various female genital infections such as vaginitis, cervicitis, pelvic inflammatory disease (PID), pyelonephritis and post-partum septicaemia. In the reproductive tract, these infections play important roles in maternal and foetal health. Genital mycoplasmas include *Mycoplasma genitalium, M. hominis, Ureaplasma parvum* and *U. urealyticum* and other co-infections including bacterial vaginosis (BV), *Chlamydia trachomatis, Neisseria gonorrhoeae* and *Trichomonas vaginalis*. Genital mycoplasmas develop point mutations in the genes of the antibiotics targets, which are responsible for increased antimicrobial resistance. Infections with these bacteria become important during pregnancy as infection may lead to infertility and foetal death. The purpose of the study was to detect the mycoplasmas and co-infections in women visiting an antenatal or reproductive biology clinic, and to determine the resistance of the mycoplasmas to antibiotics used to treat infections.

This study included self-collected vaginal swabs from 55 women attending an antenatal clinic and 51 women attending a reproductive clinic at a tertiary hospital, Pretoria, South Africa. Genital mycoplasmas were cultured on A7 agar. The Nugent scoring system was used to diagnose BV. Genital mycoplasmas were diagnosed and the antimicrobial susceptibility profiles determined with the Mycofast Revolution assay. Genital mycoplasmas were detected and speciated with a conventional multiplex polymerase chain reaction assay. In addition a real time PCR assay was used for the detection of genital mycoplasmas and other co-infections.

The overall prevalence of sexually transmitted infections (STIs) for both clinics detected by Anyplex II STI-7 showed 64.1% (68/106) of *U. parvum*, which was the most commonly identified pathogen, followed by 28.3% (30/106) of *M. hominis*, 24.5% (26/106) of *U. urealyticum*, 11.3% (12/106) of *T. vaginalis,* 5.7% (6/106) of *C. trachomatis,* 2% (2/106) *M. genitalium* and 0.9% (1/106) of *N. gonorrhoeae*). Genital mycoplasmas were detected in 67% (37/55) of samples at the antenatal clinic and 57% (29/51) of the samples at the reproductive biology clinic using the Mycofast Revolution assay. This study has shown that the genital mycoplasmas infection rate was higher among pregnant women than in the women visiting the reproductive clinic. Therefore, in order to prevent complications in pregnant women, the foetus and the neonate, screening routinely for the presence of genital mycoplasmas is recommended.

Keywords: Bacterial vaginosis, Genital mycoplasmas, Mycofast Revolution, Real time PCR, Sexually transmitted infections

CHAPTER 1

1.1 INTRODUCTION

Genital mycoplasmas are regarded as the most frequently isolated species in the genital tract and are commonly found in the genitourinary tract of pregnant and non-pregnant women (Bayraktar *et al*., 2010). Genital mycoplasmas are also the most frequent organisms found in the amniotic fluid (AF) of women with preterm premature rupture of membranes (PPROM) (Kacerovsky *et al.,* 2011). The genital mycoplasmas include *Mycoplasma genitalium*, *Mycoplasma hominis, Ureaplasma parvum* and *Ureaplasma urealyticum* (Kacerovsky *et al.,* 2011).

Mycoplasma hominis and *U. parvum* have been found as the most prevalent amongst the genital mycoplasmas and are a cause of genitourinary diseases in both men and women (Costoya *et al*., 2012). *Mycoplasma hominis* and *U. urealyticum* are most commonly found in the genital tract of women who are sexually active (Bayraktar *et al*., 2010). The colonisation rates for *M. hominis* range between 20% and 30% and for *U. urealyticum* it ranges between 60% and 80% (Bayraktar *et al*., 2010). In a study conducted in Pretoria, South Africa where vaginal swabs were collected fom pregnant women attending an antenatal clinic *Ureaplasma* species were detected in 76% (73/96) of specimens and 40% (29/73) of *Ureaplasma* positive specimens were also positive for *M. hominis* (Redelinghuys *et al*., 2014).

Mycoplasma genitalium was first isolated from men who suffered from urethritis (Waites *et al.,* 2012). *Mycoplasma genitalium* is now considered an established cause of male non-gonococcal urethritis (NGU) and female cervicitis (Waites *et al*., 2012; Hamasuna, 2013). *Mycoplasma genitalium* can cause other diseases, such as acute epididymitis, chronic prostatitis and balanoposthitis in males and can cause pelvic inflammatory disease, urethritis and adverse pregnancy events in females (Waites *et al*., 2012; Hamasuna, 2013).

Fertility in both men and women may be adversely affected due to the effect of ureaplasmas and mycoplasmas infections (La Vignera *et al*., 2011). Studies generally agree that the prevalence of ureaplasmas and mycoplasmas is higher among male partners in infertile couples than in fertile males (Friberg and Gnarpe, 1974; Andrade-Rocha, 2003 Lee *et al*., 2013). Evidence supporting both *M. hominis* and *U. urealyticum* as agents involved in infertility is not nearly as conclusive as existing evidence for pathogens such as *C. trachomatis* and *N. gonorrhoeae* (Sweet , 1986; Tyagi, 1999; Baczynska *et al*., 2005). Some investigators have been able to detect each of the organisms in infertile patients and in patients with upper genital tract disorders and several of these organisms could not be linked to infertility (Sweet, 1986; Tyagi, 1999; Baczynska *et al*., 2005). *Ureaplasma* infection can lead to disturbances in spermatogenesis, sperm function, transport and penetration as well as the death of spermatozoa (Borovkova *et al*., 2011). In women, a reduction in successful *in vitro* fertilization and endometritis have been observed associated with *Ureaplasma* infections (Borovkova *et al*., 2011). Among the *Ureaplasma* spp., *U. parvum* is the most frequently found species in the genital tract of infertile couples (Borovkova *et al*., 2011).

Bacterial vaginosis (BV) is considered the most common disturbance of the vaginal microflora in which the normally dominant lactobacilli are replaced with increased concentrations of a diverse array of bacteria (Gallo *et al*., 2012). This includes bacteria such as facultative and anaerobic organisms, including *Bacteroides* spp., *Gardnerella vaginalis*, genital mycoplasmas and *Mobiluncus* spp. (Gallo *et al*., 2012). Bacterial vaginosis is associated with sexual activity with new and multiple sex partners (male and female) (Kenyon *et al*., 2013). Bacterial vaginosis has been referred to as one of the most prevalent enigmas in the field of medicine and is associated with significant clinical sequelae including the increased risk of HIV acquisition, preterm delivery and pelvic inflammatory disease (PID) (Kenyon *et al*., 2013; Salah *et al*., 2013).

Mycoplasmas can occasionally cause severe infections in both immunocompromised and immune competent individuals; however, their identification in clinical specimens is hindered by the fact that routine microbiological methods fail to detect this group of pathogens (MacKenzie *et al*., 2010). The reference standard for the detection of the recoverable organisms like *M. hominis* and *Ureaplasma* spp. is culture, which is more laborious and less sensitive compared to molecular methods (Larsen and Hwang, 2010). A selective agar medium like A7 *Mycoplasma* agar (bioMérieux, France) can be used to distinguish between *M. hominis* and *Ureaplasma* spp. (Naher and Said, 2013). This medium combines a rich nutritive base containing peptones, horse serum and growth factors (cysteine, arginine, urea) favouring the growth of mycoplasmas (Naher and Said, 2013). Other media commonly used for cultivation of mycoplasmas include A2 agar and A8 agar (Waites *et al*., 2005).

Commercially available diagnostic kits include the Mycoplasma Duo Test Kit® (Sanofi Diagnostic Pasteur, France) and Mycofast® Screening Revolution (ELITech, France) kit (Tarrant *et al*., 2009).

Slow-growing species, such as *M. genitalium*, which makes identification by culture methods complicated and impossible, require the use of molecular methods (Waites *et al*., 2012). Molecular methods, such as PCR assays (conventional and real-time) are sensitive and allow for the rapid detection of infectious agents and do not require the presence of viable organisms (Gdoura *et al*., 2008). Commercially available multiplex PCR assays, such as the Seegene Anyplex II STI-7 detection kit, have made the testing for these pathogens more convenient as multiple causative organisms can be detected simultaneously (Choe *et al*., 2013). The Anyplex II STI-7 detection kit, a multiplex real-time PCR assay can simultaneously detect seven microorganisms (*Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Trichomonas vaginalis*, *M. genitalium*, *M. hominis*, *U. urealyticum*, and *U. parvum*) (Choe *et al*., 2013).

The gold standard laboratory diagnostic methods for bacterial vaginosis include the Nugent scoring system and the Amsel's criteria (Amsel *et al*., 1983; Nugent *et al*., 1991). Nugent scoring is done by assessing for the presence of large Gram-positive rods (*Lactobacillus* morphotypes; decrease in *Lactobacillus* scored as 0 to 4), small Gram-variable rods (*G. vaginalis* morphotypes; scored as 0 to 4), and curved Gram-variable rods (*Mobiluncus* spp. morphotypes; scored as 0 to 2) (Nugent *et al*., 1991). In 1983, Amsel *et al*. proposed the clinical criteria for diagnosing BV (Amsel *et al*., 1983). Amsel's criteria define BV as being the presence of three of the following four criteria: vaginal pH of 4.5, clue cells on saline wet mount, release of a fishy amine odor on addition of 10% KOH to a drop of vaginal discharge, and a characteristic thin, homogenous vaginal discharge (Amsel *et al*., 1983). The Ison-Hay criteria observe the ratio of bacterial morphotypes involved in bacterial vaginosis rather than the exact number (Hay *et al*., 1994; Ison *et al*., 2002; Kampan *et al*., 2011; Mancuso *et al*., 2011).

The World Health Organization (WHO) recommends that sexually transmitted infections (STIs) be managed at the first point of contact with the health services using the syndromic approach (Adler, 1996). In South Africa, the STI syndromic management approach was introduced to primary health care almost 20 years ago. South African genital tract infections (GTI) treatment guidelines employ syndromic management consistent with WHO recommendations (Kaida *et al*., 2018). Syndromic management is based on the identification of syndromes (i.e., consistent groups of symptoms and clinical signs) associated with infection by a defined pathogen, followed by syndromic treatment for the most common causative GTI organisms (Kaida *et al*., 2018).

Mycoplasmas lack a cell wall, which is the target of beta-lactam antibiotics and vancomycin, a glycopeptide (Waites *et al*., 2005). Tetracyclines, macrolides and quinolones are the major antibiotics used in the treatment of urogenital infections caused by mycoplasmas, except for pregnant women, neonates and children, for which the recommended antibiotic is erythromycin (Bayraktar *et al*., 2010). Tetracyclines form part of the first-line treatment of human urogenital infections caused by *Mycoplasma* spp. and *Ureaplasma* spp. (Degrange *et al*., 2008). High-level resistance to tetracyclines in *M. hominis* and *Ureaplasma* spp. has been associated with the presence of the *tet*M, which is the solitary tetracycline resistance mechanism acquired by human mycoplasmas (Taylor-Robinson, 2007). High-level resistance to tetracyclines in *Ureaplasma* spp. has been associated with the transposon 916 (Tn*916*)-Tn*1545* family of conjugative transposons that carry *tet*M genes (Govender and Chalkley, 2012).

Macrolides and lincosamides are antibiotics used for the treatment of *U. urealyticum* infections especially among children and those allergic to tetracyclines and quinolones (Lu *et al*., 2010). Resistance to macrolides has been widespread in *U. urealyticum*, though the molecular mechanisms of resistance to macrolides are well understood in bacteria; it remains unclear in *U. urealyticum* (Lu *et al*., 2010). Transition mutations in 23S rRNA accounted for acquired resistance to macrolides in *U. urealyticum* (Lu *et al*., 2010).

Treatment of pregnant women with BV is described as a major problem because large clinical trials have failed to demonstrate a reduction in adverse pregnancy outcomes following antibiotic therapy (Bodnar *et al*., 2009). The standard treatment for bacterial vaginosis is metronidazole, 400 mg orally 12 hourly for five to seven days (Workowski and Berman, 2006). An alternative is a 2 g single dose, or 2 g tinidazole, which is more expensive (Workowski and Berman, 2006).

The prevalence of genital mycoplasma infections over the last decade has been increasing globally, which may be due to the increase in mycoplasma antibiotic resistance or improved diagnostic methods. Currently, in South Africa especially in the Gauteng region, there is limited information available on the prevalence of mycoplasma in women visiting an antenatal or reproductive biology clinic. There is limited information available on the relationship between mycoplasmas and other co-infections, such as bacterial vaginosis, *C. trachomatis, Neisseria gonorrhoeae* and *Trichomonas vaginalis*. It is important to detect these STIs and intervene early enough to prevent complications and adverse outcomes in pregnancies in a high human immunodeficiency virus (HIV) prevalence country, such as South Africa. In pregnant women, this might minimise the risk for complications at a later stage, which may reduce the rates of neonatal morbidity and mortality. This study will therefore provide valuable new data on the prevalence rates of the mycoplasmas and other sexually transmitted co-infections.

1.2 Aim

The aim of this study was to detect the mycoplasmas in women visiting an antenatal or reproductive biology clinic, followed by the detection of co-infections in these women.

1.3 Objectives

The objectives of this study were:

- To obtain (self-collected) vaginal swabs from 100 women attending an antenatal clinic and 100 women attending a reproductive biology clinic
- To culture *Ureaplasma* spp. and *Mycoplasma hominis* using commercial media
- To determine the antimicrobial susceptibility test of *Ureaplasma* spp. and *M. hominis* using the Mycofast Revolution assay
- To diagnose bacterial vaginosis using the Nugent scoring system
- To extract genomic DNA from the swabs using a commercial kit
- To molecularly detect mycoplasmas, ureaplasmas and co-infections

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Sexually transmitted infections (STIs) represent a major public health problem worldwide (Caini *et al*., 2014). Sexually transmitted infections have been shown to increase the risk of acquisition and transmission of human immunodeficiency virus (HIV) infection, which may contribute notably to morbidity and mortality (Travassos *et al*., 2012). Bacterial sexually transmitted diseases (STDs), such as urethritis and cervicitis are important sources of morbidity in both males and females and their prevalence rates remain high worldwide (Hamasuna, 2013). Due to the physiological changes that accompany pregnancy, such as congestion of the cervix, oedema of the vaginal mucosa and alterations in the vaginal flora, pregnant women are at an increased risk of acquiring STIs (Travassos *et al*., 2012).

2.2 Brief history of mycoplasmas

Mycoplasmas are the smallest free-living Gram-positive wall-less bacteria with a wide environmental and host distribution, causing diseases in humans and in animals (Razin *et al*., 2010). In 1898, the first cultivation of *Mycoplasma*, the bovine pleuropneumonia agent, was reported (Razin *et al*., 1992; Razin, 2010). Mycoplasmas were confused with the L-form bacteria as the L-form bacteria resemble mycoplasmas in morphology, in the peculiar ‘‘fried egg’’ colony shape as well as in the lack of cell walls (can be partially or entirely) (Razin *et al*., 1992; Razin, 2010). The literature focusing on mycoplasmas in the 1950s and 1960s was filled with papers supporting or opposing the definition of mycoplasmas as bacterial L-forms (Razin *et al*., 1992; Razin, 2010). The first genomic analysis data obtained by DNA hybridisation in the late 1960s ruled out any relationship of mycoplasmas with L-form bacteria; this brought the confusion and opposing opinions to an end (Woese *et al*., 1980; Razin *et al*., 2010).

Ureaplasma spp. were originally recognised as tiny (T)-strain mycoplasmas due to their small colony size (Frølund *et al*., 2014). In 1954 ureaplasmas were first isolated from human genital samples; these bacteria were then assigned to a new genus and species *Ureaplasma urealyticum* in 1974 (Frølund *et al*., 2014). *Ureaplasma urealyticum* was originally divided into two biovars namely *Ureaplasma* biovar 2 or T980 and *Ureaplasma* biovar 1 or parvo

(Robertson and Chen, 1984). The two biovars were then assigned species status; *Ureaplasma urealyticum* (formerly biovar 2) which consists of the serotypes 2, 4, 5, 7 to 13, and *Ureaplasma parvum* (formerly biovar 1 or parvo), which consists of the serotypes 1, 3, 6 and 14 (Fanrong.*et al*., 1999; Robertson *et al*., 2002).

2.3 Characteristics of genital mycoplasmas

Genital mycoplasmas are the smallest free-living organisms with unique characteristics distinguishing the bacteria from other prokaryotes (Zhu *et al*., 2012). These characteristics include the lack of a cell wall, a small genome size, and limited biosynthetic functions (Schneider *et al*., 2015). *Ureaplasma* spp. have characteristics, which include unique surface proteins responsible for serotype specificity as well as the ability to hydrolyse urea for energy (Robertson *et al*., 2002).

2.3.1 Scientific classification of genital mycoplasmas

The mycoplasmas, which include the genera *Mycoplasma* and *Ureaplasma,* belong to the Phylum *Firmicutes* and Class *Mollicutes* (Robertson *et al*., 2002) (Table 2.1). The *Mollicutes* comprises four orders, five families and two hundred known species, with seventeen of these species known to infect humans (Waites *et al*., 2009). Genital mycoplasmas are regarded as the species most frequently isolated from the genital tract and are commonly found in the genitourinary tract of pregnant and non-pregnant women (Bayraktar *et al*., 2010). The genital mycoplasmas include two *Mycoplasma* species, which are *M. genitalium* and *M. hominis*, and two *Ureaplasma* species that include *U. parvum* and *U. urealyticum* (Kacerovsky *et al*., 2011).

Table 2.1: Scientific classification of the genital mycoplasmas (Bradbury, 2001; Robertson *et al*., 2002)

Rank	Name
Kingdom	*Bacteria*
Phylum	*Firmicutes*
Class	*Mollicutes*
Order	*Mycoplasmatales*
Family	*Mycoplasmataceae*
Genera	*Mycoplasma and Ureaplasma*
Species	*M genitalium, M hominis, U. parvum* and *U. urealyticum*

Mycoplasma genitalium is unlike other genital mycoplasmas that are common commensals in the lower urogenital tract of many healthy adults (Waites *et al*., 2012). *Mycoplasma genitalium* is less commonly detected in the absence of a clinical infection (Waites *et al.*, 2012). The bacterium possesses a terminal structure, the *M. genitalium* adhesin protein (MgPa) that facilitates its attachment to epithelial cells, spermatozoa and erythrocytes and invasion of epithelial cells with evidence of nuclear localisation (Waites *et al.*, 2012).

2.3.2 Genomics of genital mycoplasmas

Mycoplasma genitalium, *M. hominis* and *Ureaplasma* spp. have a small genome size and share the same niche (Zeighami *et al*., 2007). This species possess the smallest genomes among self-replicating free-living organisms (Pereyre *et al*., 2009). *Mycoplasma genitalium* has the smallest genome compared to other genital mycoplasmas species, comprising 580 Kb, with a capacity to encode only 482 genes (Pereyre *et al*., 2009). The genome of *M. hominis* is slightly larger than that of *M. genitalium*, as determined with pulsed-field gel electrophoresis revealing a chromosome size of 696 Kb (Pereyre *et al*., 2009). The largest sequenced genome of the three species is the one of *U. parvum* (previously called *U. urealyticum* serovar 3), encompassing 751 Kb as indicated in Table 2.2 (Pereyre *et al*., 2009).

Table 2.2: Comparison of the general features of the *M. hominis* genome with those of *M. genitalium* and *U. parvum* serovar 3 (Pereyre *et al*., 2009)

Features	***M. hominis* (PG21)**	***M. genitalium* (G37)**	***U. parvum* (serovar 3)**
Genome size (bp)	665 445	580 074	751 719
GC content of genome (%)	27.1	31.7	25.5
Gene density (%)	89.8	91.2	91.3
Total number of CDS	537	482	614
Hypothetical proteins	106	2	182
Conserved hypothetical proteins	86	164	107
CDSs with predicted function	345	316	325
Pseudo genes	14	5	0
Average protein length (aa)	369	363	371
Proteins with predicted MW > 300kDa	2	0	2
Predicted lipoprotein	43	18	37
rRNA sets	2	1	2
tRNA	33	36	30
Start codon usage (%)			
AUG	95.1	88.5	92.5
UUG	3.0	4.1	3.0
GUG	1.7	7.4	4.5
Other	0.2	0	0
Stop codon usage (%)			
UUA	83	72	85
UAG	16	27	14
Tryptophan codon usage (%)			
UGA	87	64	87
UGG	13	36	13

The general features of the *M. hominis* PG21 genome and their comparison with those of *M. genitalium*G37 and *U. parvum* serovar 3 are shown in Table 2.2 (Pereyre *et al*., 2009). The *M. hominis* PG21 genome is a single, circular chromosome of 665,445 bp with an overall G+C content of 27.1% (Pereyre *et al*., 2009).

2.3.3 Metabolic pathways used by the genital mycoplasmas

Mycoplasma hominis, *M. genitalium*, and *U. parvum* display marked species differences in their reduced energy metabolism (Pereyre *et al*., 2009). Their species-specific genes (Figure 2.1) are involved in the major energy-generating metabolic pathways (Pereyre *et al*., 2009). *Mycoplasma genitalium* produces energy through the glycolytic parthway, which has 172 specific coding sequences (CDSs) (Pereyre *et al*., 2009). Several genes encoding components of the carbohydrate, pyruvate and glycerol metabolic pathways were found among the 172 specific CDSs in *M. genitalium*, including carbohydrate transporter genes (Pereyre *et al*., 2009). Other genes that were specifically found in *M. genitalium* include the 1 phosphofructokinase encoding genes, *fru*K (MG063), *fru*A (MG062) and *pst*G (MG069) of the fructose and glucose phosphoenolpyruvate dependent sugar phosphotransferase transport system (PTS) (Pereyre *et al*., 2009).

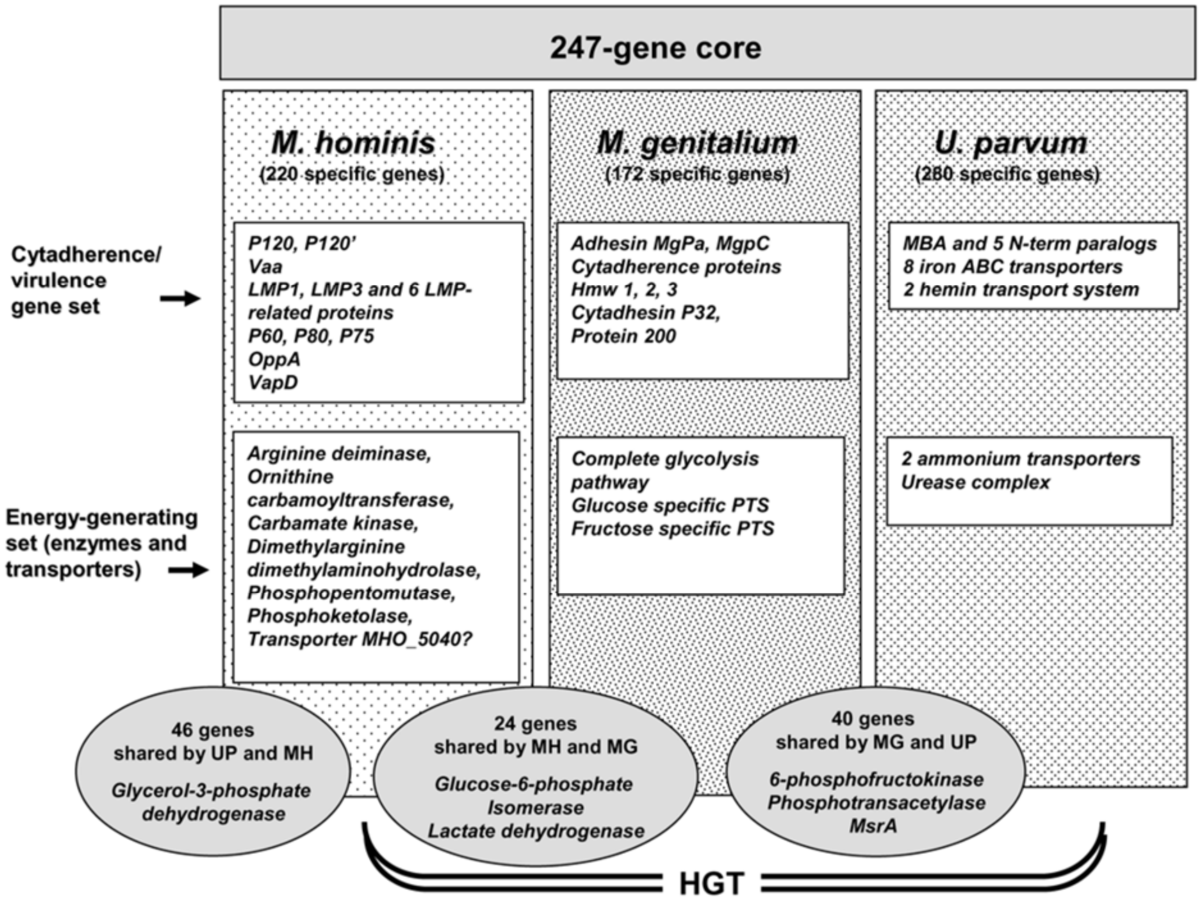

Figure 2.1: Comparative analysis of the genomes of three minimal mollicutes affecting humans: Focus on known species-specific proteins involved in cytadherence/virulence and energy-generating pathways. LMP, large membrane protein; PTS, phosphoenolpyruvate phosphotransferase system; HGT, horizontal gene transfer; UP, *U. parvum*; MH, *M. hominis*; MG, *M. genitalium* (Pereyre *et al.,* 2009)

Ureaplasma spp. generate 95% of its Adenosine Triphosphate (ATP) through the hydrolysis of urea; this characteristic is not found in the other mycoplasmas (Oshima and Nishida, 2008). In *U. parvum*, which generates ATP through urea hydrolysis, the seven components of the urease complex (UU428 to UU434), the ammonium transporters Amt-1 and Amt-2 (UU218, UU219) and the atypical delta subunits of the FoF1-ATPase (UU133, UU134) probably involved in this unique energy production pathway, were found among the 280 CDSs that were absent from *M. hominis* and *M. genitalium* (Pereyre *et al*., 2009). All the genes coding for the enzymes of the Embden-Meyerhoff-Parnas (EMP) pathway were present in *M. hominis* species, except 6-phosphofructokinase (Pereyre *et al*., 2009). The genes coding for enzymes in the pentose phosphate pathway was incomplete in *M. hominis*, with the absence of genes encoding glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase; these genes were also absent from *M. genitalium* and *U. parvum* (Pereyre *et al*., 2009).

Figure 2.2 shows an integrated view of the metabolism and transporters of *M. hominis*, *M. genitalium* and *U. parvum*. The metabolic products are shown in black. Putative proteins present in *M. hominis* are shown in green; proteins absent from *M. hominis* are shown in red. The ‘‘MG’’ in purple boxes indicates that the gene encoding the corresponding protein is present in the *M. genitalium* G37 genome. The ‘‘UP’’ in blue boxes means that the gene encoding the corresponding protein is present in the *U. parvum* genome.

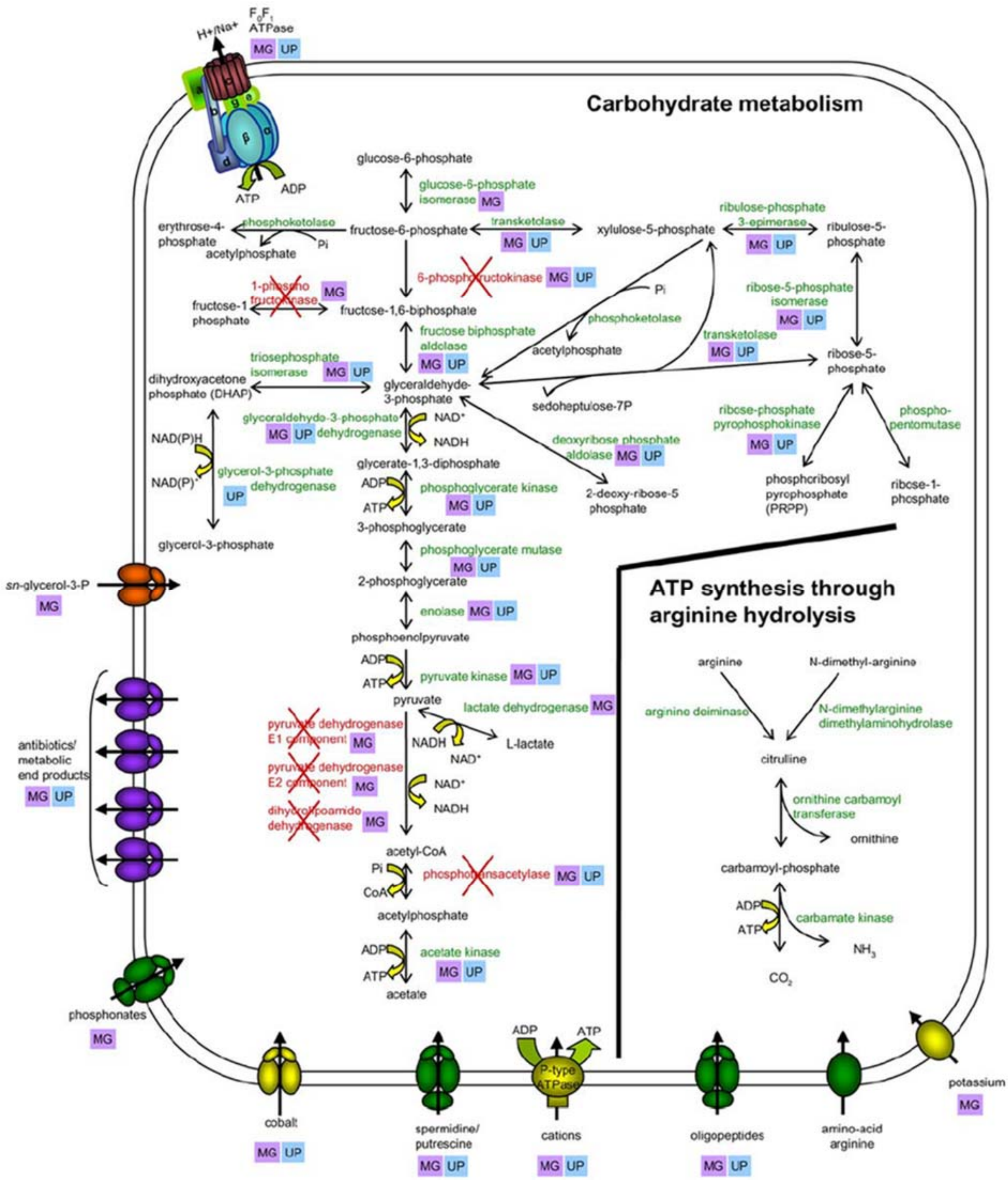

Figure 2.2: Comparison of carbohydrate and arginine-metabolism pathways and transporters encoded by *M. hominis* PG21, *M. genitalium* G37, and *U. parvum* serovar 3 (Pereyre *et al*., 2009)

Transporters are coloured according to their substrates: yellow, cations; green, anions and amino acids; orange, carbohydrates; purple, multidrug and metabolic end-product efflux. Arrows indicate the direction of substrate transport (Pereyre *et al*., 2009).

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2.4 Pathogenesis and virulence factors of genital mycoplasmas

The genus *Mycoplasma* alone contains more than 120 species at present, and new members are continuously being discovered of which several are well-established as classic pathogens that are etiological agents of disease in man and animals (Schnee *et al*., 2012). These species generally exhibit strict host and tissue specificity with a preference for the respiratory and urogenital tracts, the mammary gland, the joints and the alimentary canal (Rosengarten *et al*., 2000). Mycoplasmas are not considered highly virulent, therefore the diseases caused are rarely of the fulminant type but rather follow a slowly progressive chronic course with high morbidity and low mortality, imposing substantial health care costs and significant agronomical production losses to the economy throughout the world (Rosengarten *et al*., 2000).

Host cell damage and the resulting clinical manifestations and disease outcomes seem to be due to host immune reactions and inflammatory responses rather than to direct toxic effects of *Mycoplasma* components such as metabolic by-products like hydrogen peroxide and superoxide radicals (Rosengarten *et al*., 2000). Another possible mechanism mediating cell damage is based on the intimate contact of the mycoplasmas with their host cells, which may lead to fusion with or invasion into these cells that may affect cell function and integrity (Rosengarten *et al*., 2000).

Pathogenesis of *Ureaplasma* spp. is determined by the presence of virulence factors such as adhesins, human IgA protease, phospholipase A and C, urease, multiple banded antigen protein and hydrogen peroxide (Viscardi and Hasday, 2009). However, the existence of the interrelationships between the presence of these virulence factors in *Ureaplasma* spp. and the incidence of diseases in humans has not yet been demonstrated (Momynaliev *et al*., 2007). The difficulty in the clarification of these interrelationships may be due to macro and microgenomic heterogeneity of the bacteria (Momynaliev *et al*., 2007). It is believed that a combination of variable strain-specific genes in the *Ureaplasma* spp. with known virulence factors determine the development of pathological processes on the mucosal surface of the human urogenital tract (Momynaliev *et al*., 2007).

Adhesins allow the attachment of the bacteria to the genital and respiratory mucosal cell (Viscardi and Hasday, 2009). The attachment allows the bacteria to withstand the clearing action of urination by the host (Waites *et al*., 2005). After attachment, the bacteria primarily reside on the mucosal membrane of the urogenital tract of the host (Waites *et al*., 2005). After the bacteria have attached, immunoglobulin A (IgA) protease is secreted to cleave the IgA1 allowing the bacteria to evade the host's immune defence (Viscardi and Hasday, 2009). Inside the cell, the bacteria secrete products, such as urease, hydrogen peroxide and phospholipids, which injure the mucosal cells (Waites *et al*., 2005). Urease production by *Ureaplasma* spp. causes a breakdown of urea to ammonia and carbonate and this carbonate spontaneously decomposes to form another molecule of ammonia and carbonic acid, which results in an increase in pH (Viscardi and Hasday, 2009). An increase in extracellular pH stimulates the pathogenecity of *Ureaplasma* spp. by damaging mucosal cells (Viscardi and Hasday, 2009).

2.5 Genital mycoplasmas in urogenital infections

Mycoplasma hominis and *Ureaplasma urealyticum* are commonly found in the urogenital tracts of healthy adults (Lee *et al.,* 2013). These two mycoplasmas are the most frequently detected species among the mycoplasmas in sexually active asymptomatic young men and women (Lee *et al.,* 2013). *Mycoplasma hominis* and *U. urealyticum* are thought to play important roles in a wide spectrum of pathological conditions in both men and women (Table 2.3), which include unexplained chronic lower urinary tract symptoms, non-gonococcal urethritis, pelvic inflammatory disease (PID), pyelonephritis, chronic prostatitis, preterm labour and spontaneous abortion through infections in the urogenital organs (Baka *et al*., 2009; Farag *et al*., 2013). *Mycoplasma hominis* is also believed to be the causes of septic arthritis and postpartum fever (Cunningham *et al*., 2013). The true role of *M. hominis* and *U. urealyticum* in BV has remained somewhat controversial in the past because investigators have failed to explore the relationship between bacterial vaginosis (BV) and genital mycoplasmas (Taylor-Robinson and Lamont, 2011). The controversy was somehow resolved by Redelinghuys *et al*., 2015 in a study that described the prevalence of and associations between BV and genital mycoplasmas in women attending antenatal care in an HIV-endemic setting (Redelinghuys *et al*., 2015). Redelinghuys *et al*. (2015) reported that all the genital mycoplasmas, except *U. urealyticum*, were present in higher numbers in samples with a high Nugent score (≥7) as compared to samples with an intermediate Nugent score (4–6) where *Ureaplasma parvum* was the most commonly isolated species.

Table 2.3: Relationship between urogenital mycoplasmas and various clinical presentations, adapted from Cazanave *et al*., 2012

Disease	*M. hominis*	*Ureaplasma* spp.	*M. genitalium*
Genital infections in men			
Acute NGU	-	+	+
Chronic NGU	-	+/-	+
Post-gonococcal urethritis	-	-	+
Balanoposthitis	-	-	+/-
Epididymitis, prostatitis	-	+/-	+/-
Gynaecological infections			
Bacterial vaginosis	+	+/-	+/-
Cervicitis	-	-	+
Urethritis	-	+	+
Endometritis	+	-	+
Salpingitis	+	-	+
Pregnancy related disorders			
Chorioamnionitis	+/-	+	?
Post-partum fever	+	+	?
Ectopic pregnancy	-	-	?
Spontaneous abortion	+/-	+/-	+/-
Premature delivery	-	+	+/-
Tubular infertility	-	+/-	+/-
Neonatal infections	+	+	?
Extragenital infections			
Septic arthritis	+	+	+
Reactive arthritis	-	+	+
Other localizations	+	+	?

+ = proved relationship or cause; +/- = non-proved relationship; - = no documented relationship; ? = not determined; NGU = non gonococcal urethritis

Mycoplasma genitalium was first isolated in 1980 from the urethral specimens of two male patients suffering from non-gonococcal urethritis in the genitourinary medicine (GUM) clinic of the St Mary's Hospital, Paddington, London (Waites *et al.*, 2012). *Mycoplasma genitalium* is now considered an established cause of male non- gonococcal urethritis (NGU) and of female cervicitis (Waites *et al*., 2012; Hamasuna, 2013). *Mycoplasma genitalium* can cause other diseases, such as acute epididymitis, chronic prostatitis and balanoposthitis in males and

PID, urethritis and adverse pregnancy outcomes in females (Waites *et al*., 2012; Hamasuna, 2013).

2.5.1 Neonatal diseases caused by genital mycoplasmas

Ureaplasma spp., which include *U. urealyticum* and *U. parvum*, are regarded as the most frequently isolated microorganisms inside the amniotic cavity during pregnancy (Kasper *et al*., 2010). The neonate that is exposed may develop conditions such as abscesses, bacteraemia, chronic lung disease, meningitis and pneumonia and this may lead to an increased risk of perinatal morbidity (Waites *et al*., 2009; Kasper *et al*., 2010).

These species are also the cause of adverse pregnancy outcomes that include miscarriage, stillbirth and preterm labour (PTL), which are regarded as the major clinical concern for obstetricians and neonatologists (Kasper *et al*., 2010). Miscarriage and stillbirth are defined as the spontaneous termination of pregnancy before and after 20 weeks of gestation respectively (Frøen *et al*., 2011). Preterm labour occurs before 37 weeks gestation and is defined as uterine contractions that lead to shortening of the cervix (O'Hara *et al*., 2013). These species may also contribute to spontaneous abortion, chorioamnionitis, premature rupture of the membranes (PROM), postpartum endometritis and growth restriction (Waites *et al*., 2009; Kasper *et al*., 2010). Chorioamnionitis is an acute infection of the placental membranes, and includes both a clinical intrauterine infection and/or the histological processes (Soraisham *et al*., 2009). Preterm infants infected with these species might provoke a vigorous pro-inflammatory response in the lungs, thus, contributing to the development of chronic obstructive pulmonary disease (COPD) (Waites *et al*., 2009). Preterm infants with respiratory distress syndrome (RDS) are at risk as RDS contributes to the morbidity and mortality of preterm infants (Cultrera *et al*., 2006).

2.5.2 Genital mycoplasmas in infertile females and associated infections

Microbial agents may affect fertility by causing obstruction of male and female reproductive systems, pregnancy loss and significant effects on perinatal mortality and morbidity (Gunyeli *et al*., 2011). Infertility has an impact on 10% of the couples in the general population and research has showed that fertility in both men and women may be adversely affected due to the negative effects of *Chlamydia* and mycoplasma infections (Gunyeli *et al*., 2011; La Vignera *et al*., 2011). Studies generally agree that the prevalence of *Ureaplasma* spp. and *Mycoplasma* spp. is higher among the male partners in infertile couples than in fertile males

(Lee *et al*., 2013; Zhou *et al*., 2017). *Ureaplasma parvum* is the most frequently identified species in the genital tract of infertile couples (Borovkova *et al*., 2011). *Ureaplasma* infection can affect spermatogenesis, sperm function, transport and penetration negatively as well as lead to the death of spermatozoa (Borovkova *et al*., 2011). In women, infections with these bacteria have been linked with unexplained infertility in women, reduced pregnancy rates after *in vitro* fertilisation and endometritis (Borovkova *et al*., 2011).

2.6 Prevalence of genital mycoplasmas

Mycoplasma hominis and *U. urealyticum* are most commonly found in the genital tract of women who are sexually active and the colonisation values for *M. hominis* range between 20% and 30% and for *U. urealyticum* it ranges between 60% and 80% (Bayraktar *et al*., 2010). *Mycoplasma hominis* and *U. urealyticum* have been found as the most prevalent amongst the genital mycoplasmas and cause of genitourinary diseases in both men and women (Costoya *et al*., 2012). In Iran, a study conducted by Amirmozafari *et al*. (2009) on 210 women with genital infections found the prevalence of *Ureaplasma* spp. to be 32.3% and that of *Mycoplasma* spp. 13.3%. Another study conducted in 2006 and 2007 by Mclver *et al*. (2009) reported the prevalence rates of *M. hominis*, *M. genitalium* and *Ureaplasma* spp. in 175 sexually active Australian women to be 64%, 13.7% and 1.3% respectively. In Cuba, a study conducted by Díaz *et al*. (2013) in May 2009 through December 2010, genital mycoplasmas were detected in 63.1% of specimens (161/255). Among these, *U. urealyticum* alone was present in 68.9% (111/161), *M. hominis* alone in 4.3% (7/161) and both in 26.7% (43/161). Schlicht *et al*. (2004) who conducted a study at the University of Wisconsin—La Crosse in young adults presenting to either the Reproductive Care clinic or the Student Health Center reported the prevalence of *M. hominis*, *M. genitalium* and *Ureaplasma* spp. in 65 sexually active young adults to be 37%, 13% and 8% respectively.

It was observed that in 2 378 sexually active but non-pregnant female students, not older than 27 years, who were recruited from London universities in 2004 to 2006 the prevalence of *M. genitalium* was 3.0% (Oakeshott *et al*., 2010). This prevalence rate was similar to the 2.3% prevalence found in Danish women of age 21 to 23 years old and the 2.8% prevalence in Japanese women, but was higher than the 0.7% prevalence in pregnant women, of mean age 31 years, attending inner London general practices or the 0.8% prevalence in young adult American women (Oakeshott *et al*., 2010; Andersen *et al*., 2007; Manhart *et al*., 2007; Hamasuna *et al*., 2008; Taylor-Robinson and Lamont, 2011). In a study conducted in

Pretoria, South Africa focusing on pregnant women, *Ureaplasma* spp. were detected in 76% (73/96) of specimens and 40% (29/73) of *Ureaplasma* positive specimens were also positive for *M. hominis* (Redelinghuys *et al*., 2014).

2.7 Bacterial vaginosis: Definition and classification

Bacterial vaginosis is considered the most common disturbance of the vaginal microflora in which the normally dominant lactobacilli are replaced with increased concentrations of a diverse array of bacteria, including facultative and anaerobic species, such as *Bacteroides* spp., *Gardnerella vaginalis*, genital mycoplasmas and *Mobiluncus* spp. (Gallo *et al*., 2012). *Lactobacillus* spp. are predominantly found in the normal vaginal bacterial flora of healthy women and are believed to play a protective role in guarding the urogenital tract against infection by pathogens (Rosenstein *et al*., 1996). Lactobacilli protect the urogenital tract by the production of lactic acid, hydrogen peroxide (H_2O_2), bacteriocins, antibiotic toxic hydroxyl radicals and probiotics and in that way are able to maintain a healthy genital status (Donders, 2007; Zodzika *et al*., 2011). Lactobacilli attach to glycolipid receptors of the epithelia *via* pili that act as ligands, thereby blocking the attachment of pathogenic microorganisms to the vaginal epithelium and inhibiting their growth (Redelinghuys *et al*., 2014; Zodzika *et al*., 2011).

The four common vaginal *Lactobacillus* species include *L. crispatus*, *L. jensenii*, *L. gasseri*, and *L. iners* (Spear *et al*., 2011). *Lactobacillus crispatus* strains in particular (up to 95%) are known to produce H_2O_2, while *L. iners* strains are weak H_2O_2 producers (Antonio *et al*., 1999). Wilks *et al*. 2004 reported the production of H_2O_2 by several *Lactobacillus* spp. during pregnancy and found that *L. jensenii* and *L. vaginalis* produced the highest levels of H_2O_2 (Wilks *et al*. 2004).

2.7.1 Impact of bacterial vaginosis on pregnant women and infertility

Bacterial vaginosis is associated with sexual activity with new and multiple sex partners (male and female) (Kenyon *et al*., 2013). Bacterial vaginosis has been referred to as one of the most prevalent enigmas in the field of medicine and is associated with significant clinical sequelae including increased risk of HIV acquisition, preterm delivery and PID (Kenyon *et al*., 2013; Salah *et al*., 2013). An excessive white-grey vaginal discharge with an unpleasant odour (especially after sexual intercourse) is the most common symptom of BV (Tominaga *et al*., 2012). This malodorous discharge coats the walls of the vagina, but does not cause irritation (Tominaga *et al*., 2012). Bacterial vaginosis is a major problem worldwide in pregnant women and several studies have been conducted to determine the global prevalence of the disease (Desseauve *et al*., 2012; Salah *et al*., 2013). The frequency of BV in low-risk pregnant women with no history of preterm delivery, aged between 25 and 29 years, is not well known (Desseauve *et al*., 2012).

Despite the possibility of a relationship between stress and BV, no studies among pregnant women have clearly established a temporal relationship between psychosocial stress and the development of BV (Cammack *et al*., 2011). The role of BV in infertility is still controversial (Salah *et al*., 2013). Some studies have linked BV to pelvic inflammatory disease (PID) and tubal infertility (Hay *et al*., 1994; McGregor *et al*., 1995; Gaudoin *et al*., 1999; Ralph *et al*., 1999; Wilson *et al*., 2002; Ross *et al*., 2005); other studies disputed any relationship between BV and PID (Rodriguez *et al*., 2001; Reddy *et al*., 2004).

2.7.2 Prevalence of bacterial vaginosis

It is estimated that up to 50% of women with BV may not be diagnosed as having the infection (Tominaga *et al*., 2012). Despite treatment, 15% to 30% of women with BV experience a recurrence within one month of treatment and an overall cure rate of less than 50% after three months has been reported (Donders *et al*., 2013). While limited population-based studies exist, the National Health and Nutrition Survey in the United States reported a BV prevalence in 17 to 49 year old women of 29% (Allsworth and Peipert, 2007) and an Australian study done in a group of 17 to 25 year old women attending general and reproductive health services found a BV prevalence of 12% (Bradshaw *et al*., 2013;Vodstrcil *et al*., 2013). In the United States, the estimated frequency of BV was found to be 16% to 39% (Amsel *et al*., 1983; Meis *et al*., 1995) and in Europe the reported frequency appears lower, ranging from 1.6% to 28% (Goffinet *et al*., 2003; Svare *et al*., 2006; Desseauve *et al*., 2012). In the 2017 STI report of the National Institute for Communicable Diseases, bacterial vaginosis (BV) was reported in 405 (50.6%) of the 801 women presenting with vaginal discharge syndrome at primary healthcare facilities in South Africa between April 2014 – September 2015 (Kularatne *et al*, 2017).

2.7.3 Diagnosis of bacterial vaginosis

The gold standard methods for BV diagnosis include the Amsel's criteria and Nugent score (Amsel *et al.*, 1983;Nugent *et al.*, 1991; Rao *et al.*, 2016). Amsel's composite criteria include the presence of a homogeneous vaginal discharge, pH of the vagina being > 4.5, the presence of clue cells in wet mount of the vaginal discharge and a positive whiff test (Amsel *et al.*, 1983; Laxmi *et al.*, 2011). According to Amsel, if three of the four criteria are positive, the patient has bacterial vaginosis (Laxmi *et al.*, 2011). Nugent scoring is done by assessing for the presence of large Gram-positive rods (*Lactobacillus* morphotypes; decrease in *Lactobacillus* scored as 0 to 4), small Gram-variable rods (*G. vaginalis* morphotypes; scored as 0 to 4), and curved Gram-variable rods (*Mobiluncus* spp. morphotypes; scored as 0 to 2) (Nugent *et al.*, 1991). Another method to diagnose BV was suggested by Ison and Hay (Hay *et al.*, 1994; Ison *et al.*, 2004). It was proposed that the proportion between the *Lactobacillus* morphotypes and *Gardnerella* morphotypes in Gram stained vaginal smears rather then counting the bacteria on an ordinal scale should be used (Hay *et al.*, 1994; Ison and Hay, 2002).

Bacterial vaginosis can be detected using the OSOM BV blue test (Shujatullah *et al.*, 2010). The test is less time consuming and requires minimal technical expertise for rapid diagnosis of bacterial vaginosis (Shujatullah *et al.*, 2010). The OSOM BV blue test is based on the principle of detecting bacterial sialidase activity (an enzyme produced by bacterial pathogens such as *G. vaginalis*, *Bacteroides* spp., *Prevotella* spp., and *Mobiluncus* spp. in vaginal discharge) (Shujatullah *et al.*, 2010).

2.7.4 Treatment of bacterial vaginosis

Most low- and middle-income countries (LMICs) rely on syndromic management to guide diagnosis and treatment of STIs. 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. Syndromic management includes appropriate health seeking behaviour of infected individuals, recognition of syndromes by the health care worker, partner management (notification and treatment), behavioural counselling and condom promotion (Figure 2.3) (National Department of Health, 2015). 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, 2002).

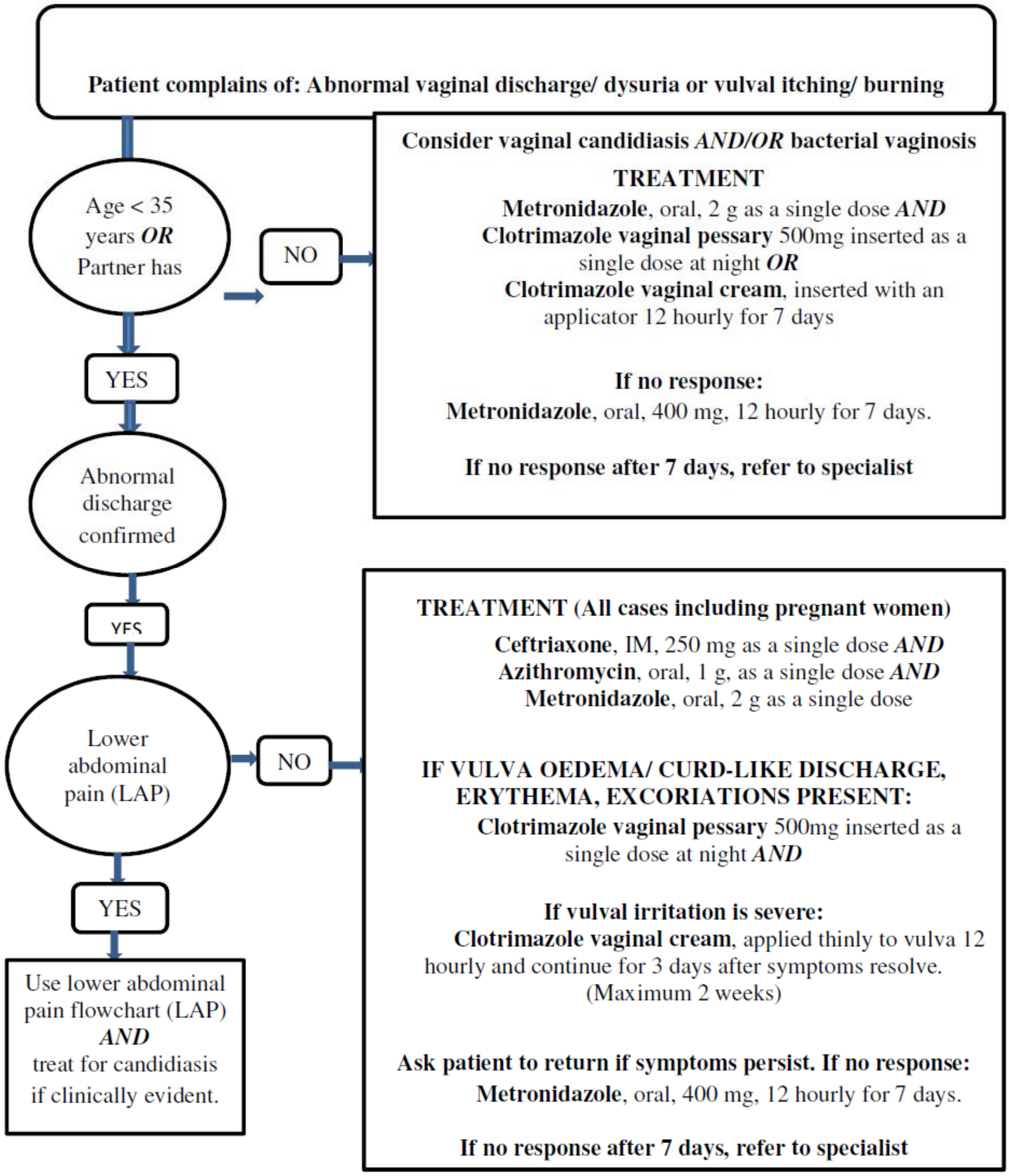

Figure 2.3: Syndromic treatment for vaginal discharge syndrome (National Department of Health, 2015)

The syndromic management approach for women presenting with vaginal discharge syndrome (VDS) at healthcare facilities has the following advantages: it is easy to use, inexpensive and it treats the patient at the first visit for most of the pathogens associated with each syndrome (Van der Eem *et al.*, 2018). However, this approach has several disadvantages, namely the majority of women with a cervical infection are asymptomatic and therefore remain untreated, whereas some symptomatic women are unnecessarily or incorrectly treated with antibiotics (Van der Eem *et al*., 2018).

2. 8. *Chlamydia trachomatis*: Definition and classification

Bacteria belonging to the genus *Chlamydia* are Gram-negative, aerobic, obligate intracellular pathogens, which are members of the order *Chlamydiales* (Grayston *et al*., 1989a; Moulder, 1991; Manavi *et al*., 2006). The genus *Chlamydia* belongs to the class *Chlamydiae* and these bacteria are characterised by their unique growth cycle and are the causative pathogens for a wide variety of diseases in humans (Grayston *et al.,* 1989; Carte *et al*., 1991). *Chlamydia trachomatis* infections are the most common bacteria that cause sexually transmitted diseases in humans and are the leading cause of infectious blindness worldwide (Carter *et al*., 1991; Everett *et al*., 1999). There are two species belonging to the genus *Chlamydophila, which* include *Chlamydophila psittaci* and *Chlamydophila pneumoniae* (Carte *et al*., 1991; Everett *et al*., 1999). *Chlamydophila pneumoniae* has been identified as the cause of pneumonia and acute respiratory disease in humans (Carte *et al*., 1991). The scientific classification of the species belonging to the family *Chlamydiaceae* is summarized in Table 2.4.

Table 2.4: The scientific classification of the *Chlamydiae* species (Grayston *et al*., 1989a; Carte *et al*., 1991; Moulder, 1991; Everett *et al*., 1999)

Kingdom	*Bacteria*
Class	*Chlamydiae*
Order	*Chlamydiales*
Family	*Chlamydiaceae*
Genera	*Chlamydia*; *Chlamydophila*
Species	*Chlamydia. trachomatis*, *Chlamydophila psittaci* and *Chlamydophila pneumoniae*

Chlamydia trachomatis infections are a major threat to the reproductive health of women and are regarded as one of the leading causes of sexually transmitted diseases (STDs) in the United States and Europe (Morré *et al*., 2000; Paavonen, 2004). *Chlamydia trachomatis* consists of the following serovars: A, B, Ba, and C which infect mainly the conjunctiva and causes ocular trachoma, which is regarded as the major cause of blindness in many inhabitants of developing countries (Morré *et al*., 2000; Paavonen, 2004; Darville, 2005). Serovars D, Da, E, F, G, Ga, H, I, Ia, J and K are predominantly isolated from the urogenital tract and are the cause of infections such as urethritis and epididymitis in men, cervicitis and salpingitis in women (Morré *et al*., 2000; Paavonen, 2004; Darville, 2005). The serovars L1, L2, and L3 are associated with lymphogranuloma venereum (LGV) (Morré *et al*., 2000; Paavonen, 2004; Darville, 2005). Lymphogranuloma venereum (LGV) primarily infects the lymphatics and can be transmitted through unprotected vaginal, anal, or oral sexual contact, which can affect both women and men having sex with men (Ceovic and Gulin, 2015).

2.8.1. Impact of *Chlamydia trachomatis* infections on pregnant women and infertility

Chlamydia trachomatis is regarded as the most common sexually transmitted bacterial infection worldwide (Cabeza *et al*., 2015). *Chlamydia trachomatis* is asymptomatic in most women and it can cause significant adverse outcomes for pregnant women as well as neonates (Cabeza *et al*., 2015). The adverse pregnancy outcomes include preterm birth, low birth weight, premature rupture of membranes, stillbirth, and miscarriage and may also cause inclusion conjunctivitis and pneumonia in neonates (Meyers *et al*., 2007; Cabeza *et al*., 2015). If *Chlamydia trachomatis* is left untreated, it can lead to complications such as pelvic inflammatory disease (PID), tubal factor infertility and ectopic pregnancy in women (Walker *et al*., 2012).

Chlamydia infection has an invasive ability, which may help in causing infertility through conditions like testicular atrophy, epididymitis, orchitis, and ductal obstruction in men (Cengiz *et al*., 1997). *Chlamydia* infection can also cause pelvic inflammatory disease, tubal occlusion, and extra-uterine pregnancy in women (Gump *et al*., 1983). *Chlamydia* infection can be found in up to 25% of the women diagnosed with tubal infertility (Khron *et al*., 1995).

2.8.2 Prevalence of *Chlamydia trachomatis*

Chlamydia trachomatis is regarded as the most prevalent bacteria among STIs (Cunningham and Beagley, 2008; Monetti *et al.,* 2013). The WHO in 2006 has estimated 100 million new cases annually of *C. trachomatis* worldwide (Land *et al*., 2009; Monetti *et al.,* 2013). The prevalence of *Chlamydia* among persons aged 14 to 39 years reported by the National Health and Nutrition Examination Survey (NHANES) using data from the cycles between 2007 and 2012 was 1.7% (Torrone *et al.,* 2014). The prevalence of *Chlamydia* among sexually active females for the age group of 14 to 24 years old (whom were the population taking part on the routine screening) in the United States for the period 2007 to 2012 was 4.7% overall (Torrone *et al.,* 2014). A prevalence 8.7% (women: 13.7%, men: 4.1%) was reported for *C. trachomatis* in a study conducted in Córdoba, Spain in asymptomatic sexually active young people and adolescents of both sexes between 18 and 24 years [221 university students (GU) and 206 non-university teenagers (GNU) with low socio-economic level] (Farinati *et al*., 2008). In Europe, three-quarters of all genital *C. trachomatis* infections are detected in the age group between 15 and 24 years old; Norway has been reported to have the third highest rate of notified *Chlamydia* cases (Gravningen *et al*., 2015).

In South Africa, there is an estimated 5.3 million people living with HIV/AIDS (PLWHA) aged 15 years and older, 340 000 of them were thought to be newly infected in 2009 (Lewis *et al*., 2012). In South Africa, the syndromic management approach is used in patients attending public facilities whom are treated for STIs either as a symptomatic individual or as a sexual contact (Lewis *et al*., 2012). Since *C. trachomatis* is one of the STI pathogens with a high rate of asymptomatic infections, screening for STIs in specific at risk groups will detect asymptomatic infections, which would otherwise remain untreated if not screened (Lewis *et al*., 2012). The 2018 STI report from the National Institute for Communicable Diseases reported *C. trachomatis* in 14.2% (114/801) women with vaginal discharge syndrome who attended primary healthcare facilities in South Africa between April 2014 – September 2015 (Kularatne *et al*, 2017).

2.9 *Neisseria gonorrhoeae*: Definition and classification

The bacteria from the genus *Neisseria* are Gram-negative, nonflagellated and cocci, occuring in pairs with adjacent sides flattened (Henriksen and Bøvre, 1968; Knapp, 1988). The genus *Neisseria* belongs to the family *Neisseriacea*e (Knapp, 1988). The family *Neisseriaceae* consists of the genera *Neisseria*, *Moraxella*, *Acinetobacter*, and *Kingella* (Henriksen and Bøvre, 1968; Knapp, 1988). The species included in the genus are *Neisseria*: *N. gonorrhoeae, N. meningitidis, N. bacilliformis*, *N. cinerea*, *N. elongata*, *N. flava* , *N. flavescens*, *N. lactamica*, *N. macacae*, *N. mucosa N. polysaccharea*, *N. sicca*, and *N. subflava* (Henriksen and Bøvre, 1968; Knapp, 1988). *Neisseria gonorrhoeae* and *N. meningitidis* are the two species that are considered pathogenic (Virji, 2009; Noinaj *et al*., 2013). *Neisseria gonorrhoeae* causes gonorrhoea, while *N. meningitidis,* colonises the nasopharynx of 10% to 20% of humans without causing any problems, but in some cases it may become invasive and spread, leading to diseases such as meningococcemia and meningitis (Virji, 2009, Noinaj *et al*., 2013).

2.9.1 Impact of *Neisseria gonorrhoeae* infections on pregnant women and infertility

Gonorrhoea is regarded as one of the most common STDs in both males and females (Kushnir *et al*., 2012; Luo *et al*., 2015). However, if this infection is left untreated it may lead to complications, such as urethritis in men and endocervicitis in women (Kushnir *et al*., 2012; Luo *et al*., 2015). *Neisseria gonorrhoeae* may also cause complications, such as cervicitis, pelvic inflammatory disease, ectopic pregnancy, neonatal conjunctivitis, and infertility in women (Kushnir *et al*., 2012). *Neisseria gonorrhoeae* also increases the risk of contracting HIV if exposed to the virus through sexual contact (Kushnir *et al*., 2012; Luo *et al*., 2015).

2.9.2 Prevalence of *Neisseria gonorrhoeae*

The gonorrhoea burden has decreased in the United States (Bradley and Satterwhite, 2012; Torrone *et al*., 2013). However, in 2009, gonorrhoea was still regarded as the second most common reported notifiable disease in the US nationally with 301 174 cases reported (Bradley and Satterwhite, 2012; Torrone *et al*., 2013). The prevalence of *N. gonorrhoeae* among female sex workers conducted between 2005 and 2013 in China was found to be 1.8% to 37.8% in different regions across the country (Luo *et al*., 2015). The prevalence of gonococcal infection in South London in a study that was conducted between January 2004 and December 2005 in community sexual and reproductive health clinics and participating general practices was found to be 4.1% (Rao *et al*., 2008). The participants in the London study were all below the age of 25 years and the prevalence was determined using nucleic acid amplification tests (NAATs) (Rao *et al*., 2008). In Ethiopia, a cross-sectional study was conducted from 1 December 2010 to February 20, 2011 at the Gynaecology outpatient department (OPD) and Microbiology Laboratory of the Hawassa Referral Hospital (Hailemariam *et al*., 2013). The gonococcal infection prevalence among the 215 participants of the Ethiopian study was found to be 5.1% (11/215) (Hailemariam *et al*., 2013). Among the 11 cases found to be positive for gonococcal infection, the numbers were almost equal for rural [54.5 % (6/11)] and urban areas [45.5% (5/11)] (Hailemariam *et al*., 2013).

2.10 *Trichomonas vaginalis*: Definition and classification

Three species of trichomonads are considered human parasites (Table 2.5): *Trichomonas vaginalis* (found in the urogenital tract), *T. tenax* (localised to the oral cavity) and *Pentatrichomonas hominis* (Maritz *et al*., 2014). The closely related protozoan, *Dientamoeba fragilis* is located in the digestive tract (Maritz *et al*., 2014).

Table 2.5: The scientific classification of the *Trichomonas vaginalis* (Schwebke and Burgess, 2004)

Rank	**Name**
Phylum	*Zoomastigina*
Class	*Parabasalia*
Order	*Trichomonadida*
Family	*Trichomonadidae*
Genus	*Trichomonas*
Species	*Trichomonas vaginalis, Trichomonas tenax, Tritrichomonas foetus, Pentatrichomonas hominis Trichomonas gallinae*

Trichomonas vaginalis is the most commonly studied parasite of all the trichomonads (Honigberg *et al*., 1964; Petrin *et al.,* 1998). This urogenital pathogen differs in size and shape, with the average length and width being 10 and 7 µm respectively (Honigberg *et al*., 1964). *Trichomonas vaginalis* is considered as an early-diverging parabasalid protozoan that happen to have branched before protozoan genera such as kinetoplastids, which are some of the earliest protozoa with mitochondria (Honigberg *et al*., 1964; Petrin *et al*., 1998; Schwebke and Burgess, 2004). *Trichomonas vaginalis* is a flagellated protozoan having five flagella, of which four are located at its anterior portion (Figure 2.4). The fifth flagellum is incorporated within the undulating membrane of the parasite which is supported by a slender non-contractile costa (Arroyo *et al*., 1993).

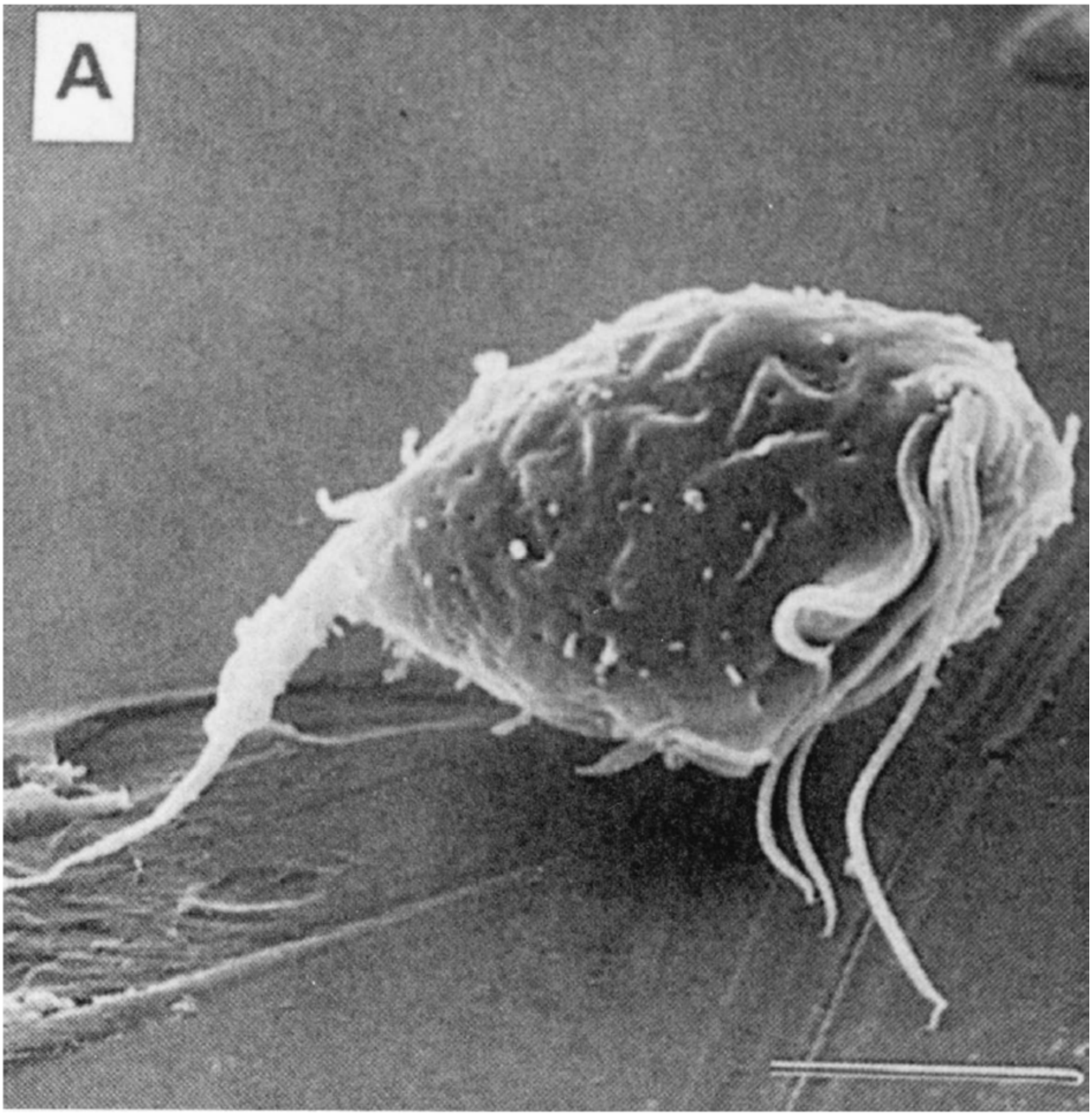

Figure. 2.4. *Trichomonas vaginalis* parasite as seen in broth culture. The axostyle, undulating membrane and flagella are clearly visible (Arroyo *et al*., 1993)

In addition, several trichomonad species are of veterinary importance, such as the avian pathogens *Trichomonas gallinae*, *Tetratrichomonas gallinarum*, and *Histomonas meleagridis***.** *Tritrichomonas foetus*, the causative agent of a venereal disease in cattle (Maritz *et al*., 2014)**.**

2.10.1 Impact of *Trichomonas vaginalis* infections onpregnant women and infertility

Trichomoniasis, which is caused by the protozoan *T. vaginalis* is the most common curable STI worldwide (Crucitti *et al*., 2003; Mavedzenge *et al*., 2010; Goo *et al*., 2016). The infection is often asymptomatic in both females and males (Crucitti *et al*., 2003; Mavedzenge *et al*., 2010; Goo *et al*., 2016). *Trichomonas vaginalis* may cause complications such as vaginitis in women and urethritis in men (Crucitti *et al*., 2003; Mavedzenge *et al*., 2010; Goo *et al*., 2016). In women, it has been associated with PID, ectocervicitis, urethritis, PROM, endometritis, and adverse pregnancy outcomes (Crucitti *et al*., 2003; Mavedzenge *et al*., 2010; Goo *et al*., 2016). *Trichomonas vaginalis* has been suggested to play a role in increasing the risk of co-infection to 1.5 to 3-fold for HIV transmission in people infected with trichomoniasis (Shafir, 2009). In a longitudinal study conducted in Zaire, Laga *et al*. (1993) reported an increased risk of HIV acquisition among women infected with *T. vaginalis*.

2.10.2 Prevalence of *Trichomonas vaginalis*

Trichomonas vaginalis is the most prevalent curable STI globally with an estimated prevalence of about 276 million new cases annually worldwide (Poole *et al*., 2013; Goo *et al*., 2016). The prevalence rates of *T. vaginalis* vary and several factors contribute to this difference in prevalence (Poole *et al*., 2013). Factors affecting the prevalence include the population and geographical region studied (Poole *et al*., 2013). The rate is generally higher among women in comparision to men (Poole *et al*., 2013). The WHO has estimated that 498.9 million new cases of trichomoniasis occurred worldwide in 2008 (Choe *et al*., 2013). The reported prevalence of trichomoniasis in the Republic of Korea was found to be 2.4% (Choe *et al*., 2013). In a cross-sectional study of 604 women conducted at 25 primary healthcare facilities in rural South Africa (Mopani district), *T. vaginalis* was detected in 20% (de Waaij *et al*., 2017).

2.11 Laboratory diagnosis of genital mycoplasmas

Mycoplasmas can be detected using phenotypic and genotypic methods. The most commonly used method for the detection of *Ureaplasma* spp. is culture, including both liquid and solid media (Pinna *et al*., 2006). However, because *Ureaplasma* spp. are fragile and fastidious, culture methods are difficult to perform and take up to 5 days (Pinna *et al*., 2006). Growth is optimal at 37°C in an anaerobic atmosphere of nitrogen with 5% carbon dioxide (CO_2) (Taylor-Robinson and Jensen, 2011). Culture of *M. genitalium* is extremely difficult, as the organism is fastidious and may require one to two months to grow in tissue culture (Nelson *et al*., 1998). Molecular methods have been developed to solve the problems encountered with culture, and have been shown to be sensitive and rapid (Nelson *et al*., 1998).

2.11.1 Culturing of mycoplasmas

Genital mycoplasma infections are commonly diagnosed by culture, which is considered as the gold standard (Stellrecht.*et al*., 2004). However, it is costly to use culture because it requires special media and expertise (Stellrecht *et al*., 2004). Culture is also time consuming and labour intensive because it can take two to five days to obtain results for *M. hominis* and *Ureaplasma* spp. Culturing of genital mycoplasmas species is based on growth in Shepard's U9 broth and growth on Shepard's A2, A7 and A8 agar media (Waites *et al*., 2005).

The specimen is first grown in Shepard's U9 broth, which consist of supplements such as horse serum as a source of sterols, yeast extracts, urea (substrate), antibiotics and antifungals to inhibit other microorganisms (Shepard, 1954). The pH indicator that the U9 broth contains is phenol red (Shepard, 1954). When the U9 broth with *Ureaplasma* spp. is incubated at 36ºC for 48 hours, the urease in the bacteria hydrolyses urea in the broth to ammonia (NH_3), which makes the media alkaline and changes the colour from yellow to pink or red due to the presence of the phenol red indicator in the media (Pinna *et al*., 2006). If the urea in the medium is depleted fast by the *Ureaplasma* spp., it may lead to the death of the bacteria (Pinna *et al*., 2006). Broth culture should be examined for colour changes twice a day for up to seven days because of the rapid death phase of the bacteria in the culture media (Pinna *et al*., 2006).

It is therefore important to subculture the bacteria on media (Shepard's A2, A7 and A8 agar media), which further support the growth (Abele-Horn *et al*., 1997; Pinna *et al*., 2006). The growth on agar media is examined for small, irregular (roughly spherical) fried-egg in appearance colonies, which grow downward into the agar and which can be observed by using a stereomicroscope at 20X or 60X magnification (Figure 2.5) and (Figure 2.6) (Waites *et al*., 2005)

Figure 2.5: Colonies of *Mycoplasma hominis* growing on A8 agar after 72 h of incubation as they appear under 126x magnification using a stereomicroscope. Colonies are typically 200 to 300 µm in diameter and demonstrate a characteristic fried-egg appearance (Waites *et al*., 2005)

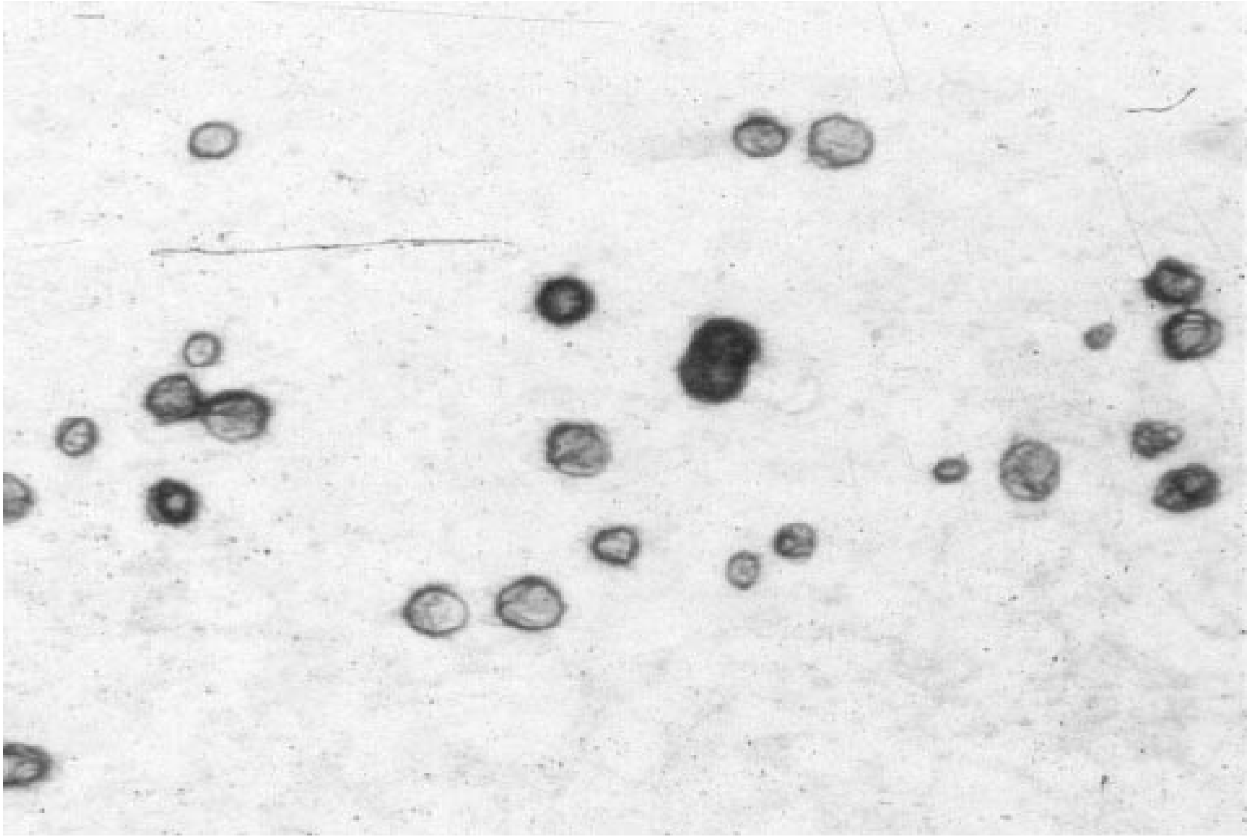

Figure 2.6: Subsurface granular colonies of *Ureaplasma* spp on A8 solid media (Waites *et al*., 2005)

Mycoplasma hominis and *U. urealyticum* can be cultured together on A7 agar media, which supports the growth of both species (Figure 2.7) (Donders *et al*., 2009). The specimen is first grown in urea–arginine broth (bioMérieux, Lyon, France) (Plečko *et al*., 2014). The urea–arginine broth also contains phenol red as a pH indicator (Donders *et al*., 2009; Plečko *et al*., 2014). When the urea-arginine broth with mycoplasmas is incubated in 5% CO_2 at 37ºC for 48 hours, the urea can be cleaved by urease for *U. urealyticum* releasing NH_3; the arginine is cleaved by the arginase of *M. hominis* releasing NH_3 (Donders *et al*., 2009; Plečko *et al*., 2014). Positive (phenol red) samples are plated on A7 agar. The A7 agar plates are incubated at 37°C in 5% CO_2 for 5 days and examined microscopically for the appearance of typical mycoplasma colonies (Plečko *et al*., 2014).

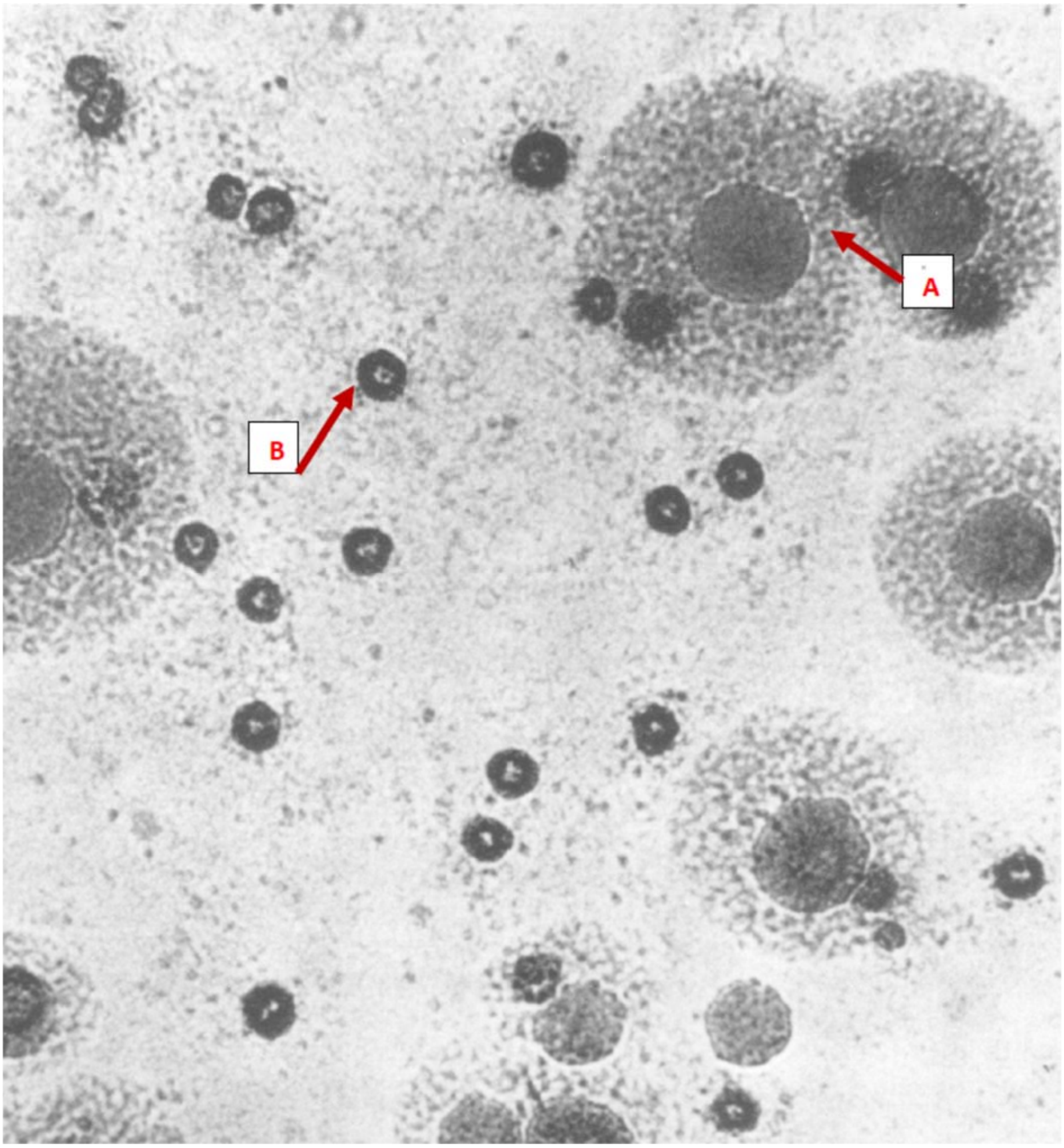

Figure 2.7: *Mycoplasma hominis* and *U. urealyticum* on A7 agar with the colonies represented by (A) the "fried-egg" morphology of *M. hominis* colonies and (B) the sea urchin" shape of *U. urealyticum* colonies (x160) (Kundsin *et al.*, 1978)

Contamination of cultures with other microorganisms is very common and culture requires constant observation with stereomicroscopy (Petrikkos *et al.*, 2007). Culture can only provide a positive or negative result and for speciation or serovar determination, as in the case of *Ureaplasma* spp., molecular techniques are needed (Juhász *et al.*, 2011).

2.11.2 Commercial kits for growth of genital mycoplasmas

Commercial diagnostic test kits for the detection, quantification and susceptibility testing of *Mycoplasma* spp. and *Ureaplasma* spp. have become available and have made the routine mycoplasmas laboratory diagnosis much easier compared to culturing methods (Govender *et al*., 2010). Some of the available kits are the Mycofast Revolution (ELiTech Diagnostic, France), Mycofast EvolutioN 3 (bioMérieux, France), MycoView (Zeakon diagnostics, France) and Mycoplasma Duo Kit (Bio-Rad, France).

The Mycofast Revolution (ELiTech Diagnostic, France) assay is a commercial assay, which provides easy detection, identification, enumeration and antibiotic susceptibility of *Ureaplasma* spp. and *M. hominis* within 24 h to 48 h (Redelinghuys *et al*., 2013). This assay is a liquid-based method based on the ability of. *M. hominis* and *Ureaplasma* spp. to metabolise urea and arginine respectively (Redelinghuys *et al*., 2013). The kit 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) (Figure 2.8) (Redelinghuys *et al*., 2013). Growth is indicated by a colour change of the medium by the pH indicator (phenol red) after 24 h to 72 h of incubation. The medium remains yellow when there is no growth of genital mycoplasmas.

Figure 2.8 Mycofast Revolution strip positive results are indicated by a pink colour, while negative results are indicated by a yellow colour

The MycoDuo (Bio-Rad, France) kit enables the identification, culture and differential titration of *Ureaplasma* spp. in urogenital and neonatal respiratory specimens (Cheah *et al*., 2005). The kit is based on the urea and arginine metabolism and the results are indicated by a colour change from yellow to pink due to the pH indicator in the medium (Figure 2.8) (Cheah *et al*., 2005; Govender *et al*., 2010). The results are read within 24 h to 48 h; this kit does not perform susceptibility testing (Cheah *et al*., 2005; Govender *et al*., 2010).

Figure 2.9 (a)

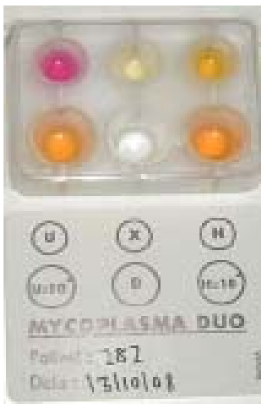

Figure 2.9 (b)

Figure 2.8: The MycoDuo kit: with 2.8 (a) showing a *Ureaplasma* spp. positive result at a high titre and 2.8 (b) showing a *Ureaplasma* spp. positive result at a low titre (Govender *et al*., 2010)

Mycoview kits (Zeakon Diagnostics, France) enable the identification and differential titration of some species of urogenital mycoplasmas, which includes *Mycoplasma hominis* and *Ureaplasma* spp. (Fouzia *et al*., 2014). This kit contains strips that provide information on the presence or absence of *Mycoplasma hominis* and *Ureaplasma* spp. (Aydogan *et al*., 2014; Fouzia *et al*., 2014). It also provides additional information on antibiotic susceptibility to azithromycin, doxycycline, josamycin, minocycline, norfloxacin, ofloxacin and roxithromycin (Fouzia *et al*., 2014). The kit is based on specific metabolic properties of the mycoplasmas (Aydogan *et al*., 2014; Fouzia *et al*., 2014). This include the metabolism of urea and arginine as well as the natural resistance of *U. urealyticum* and *M. hominis* to lincomycin and erythromycin respectively. The test strips are evaluated following incubation of 24 h for *U. urealyticum* and 48 h for *M. hominis* (Aydogan *et al*., 2014).

2.11.3 The detection of genital mycoplasmas using molecular methods

Slow-growing species, such as *M. genitalium*, which makes identification by culture methods complicated and impossible, necessitated the development of molecular methods (Waites *et al*., 2012). Molecular methods, such as polymerase chain reaction (PCR) assays (conventional and real-time) are sensitive and allow for the rapid detection of infectious agents and do not require the use of viable organisms (Gdoura *et al*., 2008). These methods are able to differentiate *Ureaplasma* spp. and serotypes, which the commercial diagnostic test kits like the new Mycofast Revolution assay are unable to do (Waites *et al*., 2005). Molecular assays are more frequently used in research laboratories than diagnostic laboratories in South Africa. This is due to the high cost of molecular assays compared to traditional culture methods.

2.11.3.1 Conventional PCR assays for detection of genital mycoplasmas

Conventional PCR assays are a type of PCR assay, which include end-point analysis of the amplicons and are detected by gel electrophoresis to determine specific fragment sizes (Evans *et al*., 2007). Conventional PCR assays detect and identify genital mycoplasmas targeting the following gene for *M. hominis*: 16S rRNA (Naher and Said, 2013). *Ureaplasma* spp. detection is done by targeting the following genes: 16S rRNA gene and the 16S-23S rRNA intergenic spacer region (Kong *et al*., 2000; Cultrera *et al*., 2006), the urease gene and the multiple banded antigen gene (*mba*) (Robertson *et al*., 2002; Naher and Said, 2013). The *Mycoplasma genitalium* adhesin protein (MgPa) gene is used as a frequent target in PCR assays to detect *M. genitalium* but some have used the 16S rRNA gene (Le Roux and Hoosen, 2010; Waites *et al*., 2012). Conventional PCR assays have been widely investigated for the detection of *Ureaplasma* spp., with sensitivity values ranging from 90% to 95% and in every investigation PCR positive, culture negative results were found (Colaizy *et al*., 2003). All of the PCR-based methods reported so far have lacked the capacity for the complete characterisation of all 14 *Ureaplasma* serotypes (Xiao *et al*., 2010). However, Xiao *et al.* (2010) have proven that the multiple-banded antigen (*mba*), a larger gene with many variations in different serotypes, can successfully serotype all *Ureaplasma* serotypes. Xiao *et al*. (2010) concluded that the *mba* gene is a better gene target compared to the urease and 16S rRNA genes for serotype-specific PCR assays.

2.11.3.2 Real-time PCR assays for detection of genital mycoplasmas

A real-time PCR assay is a type of PCR that quantify DNA and RNA products (Baczynska *et al*., 2004). This assay offers interesting advantages such as rapidity, performed in a closed system, which eliminates the risk of carry-overs, real-time monitoring of PCR activity, quantification of amplification product and, if required it can also perform mutation analysis (Baczynska *et al*., 2004). In real-time quantitative PCR assays, the PCR products are monitored and analysed during every cycle as it accumulates (Loens *et al*., 2010). Absolute quantification is achieved by using serially diluted standards of recognised concentrations to produce a standard curve (Loens *et al*., 2010).

Genital mycoplasmas can be detected simultaneously with other STIs using multiplex real-time PCR assays (Choe *et al*., 2013). The commercially available multiplex real-time PCR kit, Anyplex™ II STI-7, can amplify seven microorganisms (*C. trachomatis*, *N. gonorrhoeae*, *T. vaginalis*, *M. genitalium, M. hominis*, *U. urealyticum* and *U. parvum*) simultaneously (Choe *et al*., 2013). This real time PCR assay is performed using the CFX96 real-time PCR system (Bio-Rad, Hercules, CA, US) (Kim *et al*., 2014). The results of this assay is analysed with the Seegene software (Seegene), which interpret the results as either positive or negative (Kim *et al*., 2014). These multiplex PCR assays have made it easier for laboratory workers and clinicians in many clinical fields to test for multiple causative organisms simultaneously (Choe *et al*., 2013). These type of assays allows for faster detection of microorganisms and a reduction in labour (Choe *et al*., 2013).

2.12 Treatment of genital mycoplasmas

Mycoplasmas lack a cell wall, which is the target of beta-lactams and glycopeptides (Waites *at al*., 2005). Tetracyclines, macrolides and quinolones are the major antibiotics used in the treatment of urogenital infections caused by mycoplasmas, except for pregnant women, neonates and children, for which the recommended antibiotic is erythromycin (Bayraktar *et al*., 2010). However, erythromycin does not penetrate the amniotic sac effectively; therefore, ureaplasmas are not eradicated from the vagina or cervix by this antibiotic (Rahman *et al*., 2016). Newer macrolides such as azithromycin and clarithromycin allow for better tolerability and the once daily dosing benefit can increase compliance (Rahman *et al*., 2016). Treatment with azithromycin is as successful as erythromycin, but with less side effects (Rahman *et al*., 2016).

Ureaplasmas are susceptible to macrolides, streptogramin combinations and ketolides but intrinsically resistant to lincosamides (Bébéar and Kempf, 2005). Doxycycline, tetracycline (in adults) and erythromycin (pregnant women and neonates) remain the antibiotic of choice for the treatment of *Ureaplasma* spp. (Waites *et al*., 2008). Tetracycline is, however, associated with bone and teeth deposition during calcification, whereas chloramphenicol causes bone marrow toxicity, therefore, the doses of these antibiotics should be monitored (Mims *et al*., 2004). Fluoroquinolones, such as ciprofloxacin have less side effects than intravenous erythromycin (Aujard *et al*., 2005), but the high level of quinolone resistance in *Ureaplasma* is a major problem, which makes ciprofloxacin not a good antibiotic for treatment (Waites *et al*., 2009).

2.13 The determination of antimicrobial resistance of genital mycoplasmas

Bacteria resist antimicrobials by certain mechanisms that include intrinsic (natural) resistance, changing the cell membrane, which leads to impermeability and active efflux of the antibiotics (Mims *et al*., 2004). The bacteria can also change the antibiotic target, produce enzymes that inactivate the antibiotic and change the metabolic pathway (Ryan and Ray, 2004).

Tetracyclines belong to the first-line treatment regimen of human urogenital infections caused by *M. hominis* and *Ureaplasma* spp. (Degrange *et al*., 2008; De Francesco *et al*., 2013). The antibiotic acts as a protein synthesis inhibitor (Degrange *et al*., 2008; De Francesco *et al*., 2013). It inhibits the binding of aminoacyl tRNA to the mRNA-ribose complex (Mims *et al*., 2004). Roberts and Kenny (1986) first described *Ureaplasma* resistance to tetracycline, their study included seven Seattle isolates from 1970 to 1972, 43 Seattle isolates from 1976 to 1984, one Los Angeles isolate, and *Ureaplasma* spp. serovars. Resistance to tetracyclines in *M. hominis* and *Ureaplasma* spp. is increasing (Degrange *et al*., 2008; De Francesco *et al*., 2013). This resistance is a consequence of the presence of the *tet*(M) determinant, which is the sole tetracycline resistance mechanism acquired by clinical isolates of human mycoplasmas (Degrange *et al*., 2008; De Francesco *et al*., 2013).

Fluoroquinolones are a synthetic class of antibiotics, that inhibit the activity of DNA topoisomerase (gyrase), the enzyme involved with the de-coiling, causing negative supercoiling of the DNA and sealing of gaps of bacterial DNA during replication (Mims *et al*., 2004). Bacterial resistance to fluoroquinolones results from amino acid substitutions at Asp112Glu in the gyrase A gene (*gyr*A) gene and Ala125Thr, Ala136Thr, Ser83Leu and Asp82Asn in *par*C (Duffy *et al*., 2006; Beeton *et al*., 2009). In a study done by Beeton *et al*. (2009) a mutation on the L4 protein caused by a six base pair (bp) deletion led to the loss of arginine and glutamine at residue numbers 66 and 67 and this was found on an isolate with high erythromycin resistance. Presence of methyltransferase enzyme modifications on macrolides has not yet been identified in ureaplasmas (Beeton *et al*., 2009).

2.14 Summary

Genitourinary infections, including STDs, are caused by a large number of diverse microbial agents that cause considerable morbidity and mortality worldwide (Amin *et al.,* 2007). The vaginal discharge syndrome and male urethritis syndrome are the most common STI syndromes diagnosed in women and men (Mhlongo *et al.,* 2010). Genital mycoplasmas are the smallest, free-living, self-replicating prokaryotic cells with a double-stranded DNA genome (Harvey *et al*., 2007). Genital mycoplasmas are part of the Gram-positive bacteria; however, these bacteria lack a cell wall, which means when a Gram-stain is performed it will take on the colour of the counterstain rather than the crystal violet (Kong and Gilbert, 2004). *Mycoplasma hominis* and *U. urealyticum* have been the topic of debates for several decades among experts in genital tract infectious disease; with indications that the former can be part of the normal flora of sexually experienced women and both may play a role in chorioamnionitis, salpingitis, BV, and postpartum endometritis (Kong and Gilbert, 2004).

Urethritis occurs in both men and women but often is unrecognised in women (McKenchnie *et al.,* 2009). It is broadly categorised as non-gonococcal urethritis or gonococcal urethritis and it is characterised by discharge and dysuria (Tsai and Li, 2013). The pathogens responsible for vaginal discharge syndrome and urethritis include *C. trachomatis, M. genitalium N. gonorrhoeae* and *T. vaginalis*, whilst the role of *U. urealyticum* in these syndromes remains minimal (Mhlongo *et al.*, 2010).

Detection of genital mycoplasmas in clinical specimens rely on specific culture techniques (Mallard *et al*., 2005). Although culturing is considered as the "gold standard", it has limitations (Stellrecht *et al*., 2004). Cultivating genital mycoplasmas in the laboratory is time-consuming (two to five days for growth), lacks sensitivity and requires repeated microscopic observations (Teng *et al*., 1994). It is a relatively expensive technique because it requires special media, a high degree of expertise and special handling of specimens to maintain viability of the genital mycoplasmas and is at risk of contamination with other organisms and it is labour intensive (Dhawan *et al*., 2006).

Rapid and reliable tests are required for the early detection of STDs to prevent adverse pregnancy outcomes and neonatal infections. Multiplex PCRs were designed for the simultaneous detection of multiple microbial pathogens in the urogenital tract (Otgonjargala *et al*., 2017). 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 PrestoPlus CT/NG/TV assay (Le Roux *et al*., 2010; Gaydos *et al*., 2013; Gueye *et al*., 2014, de Waaij *et al*., 2015).

The prevalence of genital mycoplasma infections over the last decade has been increasing globally, which is due to the increase in mycoplasma antibiotic resistance. Currently, in South Africa especially in the Gauteng region, there is limited information available on the prevalence of mycoplasmas in women visiting an antenatal or reproductive biology clinic. There is limited information available on the relationship between mycoplasmas and other co-infections, such as BV*, C. trachomatis, N. gonorrhoeae* and *T vaginalis*. It is important to detect these STIs and intervene early enough to prevent complications and adverse outcomes in pregnancies in a high HIV-prevalence country, such as South Africa. In pregnant women this might minimise the risk for complications at a later stage, which may reduce the rates of neonatal morbidity and mortality.

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CHAPTER 3

DETECTION AND ANTIMICROBIAL SUSCEPTIBILITY PROFILES OF GENITAL MYCOPLASMAS IN WOMEN VISITING AN ANTENATAL AND REPRODUCTIVE BIOLOGY CLINIC OF AN ACADEMIC HOSPITAL, PRETORIA, SOUTH AFRICA

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

3.1 Abstract

Genital mycoplasmas are opportunistic bacteria that are associated with undesirable gynaecologic and reproductive events. These bacteria are characterised by their small size, lack of cell wall, extremely fastidious *in vitro* environmental requirements, and tendency to form centred colonies on solid media. Ureaplasmas and *M. hominis* are considered opportunistic pathogens because they can be isolated from the lower urogenital tract of healthy women as well as from individuals with disease. The commercial Mycofast Revolution assay permits the phenotypic detection and identification of genital mycoplasmas. This assay allows for antimicrobial susceptibility testing with specific minimum inhibitory concentrations (MICs) as defined by the 2011 Clinical and Laboratory Standards Institute (CLSI) guidelines. The antibiotics that are tested include clindamycin, erythromycin, levofloxacin, moxifloxacin, and tetracycline (CLSI, 2011). The aim of this study was to detect genital mycoplasmas as well as their antimicrobial susceptibility profiles in women visiting an antenatal and the reproductive biology clinic of a tertiary academic hospital in Pretoria, South Africa.

Methods: Women who attended either the antenatal or the reproductive biology clinic at an academic hospital in Pretoria, Gauteng South Africa, who met the inclusion criteria, were approached to participate in this study. Four self-collected vaginal swabs were obtained from each participant visiting the clinics who gave signed informed consent. The first swab was used to inoculate A7 *Mycoplasma* agar plates for culturing genital mycoplasmas. The second swab was used to perform Nugent scoring for diagnosing bacterial vaginosis. The third swab was used to inoculate the Mycofast Revolution assay for the identification, enumeration and antimicrobial susceptibility profiles of genital mycoplasmas and the fourth swab was used for a molecular conventional PCR assay for the detection of genital mycoplasmas.

Results: Hundred and six participants who either attended the antenatal (55) or reproductive biology clinic (51) at an academic hospital in Pretoria, Gauteng South Africa were recruited.

In the reproductive biology clinic, 53% (27/51) of cultures were positive for *Ureaplasma* spp., and 18% (9/51) for *M. hominis*. *Ureaplasma* spp. resistant to tetracycline in the antenatal and the reproductive biology clinics were 51% (18/35) and 52% (14/27) respectively. Bacterial vaginosis was diagnosed using the Nugent scoring system and 20% (11/55) of the participants were BV positive in the antenatal clinic and 20% (10/51) in the reproductive biology clinic. The Mycofast Revolution assay detected genital mycoplasmas in 67% (37/55) of samples at the antenatal clinic and 57% (29/51) in the reproductive biology clinic. In the antenatal clinic, 64% (35/55) of cultures were positive for *Ureaplasma* spp., and 27% (15/55) for *M.* hominis. The overall prevalence of *M. genitalium*, *M. hominis*, *U. parvum* and *U. urealyticum* in both clinics combined detected using conventional multiplex PCR assay was 1% (1/106), 5% (5/106),19% (20/106) and 4% (4/106) respectively. *Mycoplasma hominis* resistance to tetracycline in the antenatal and the reproductive biology clinics was 56% (5/9) and 51% (18/35) respectively.

Conclusion: This study has shown that the infection rate of genital mycoplasmas was higher among pregnant women. In order to prevent complications in pregnant women, the foetus and the neonate, routine screening for the presence of genital mycoplasmas is strongly recommended. This study has shown that the Mycofast Revolution assay could be considered as a cost-effective alternative to conventional culture methods for the rapid detection of genital mycoplasmas as well as antibiotic resistance.

3.2 Introduction

Genital mycoplasmas are found in the genital milieu of up to 80% of pregnant and non-pregnant women (Rahman *et al*., 2016). Genital mycoplasmas consist of *Mycoplasma hominis*, *M. genitalium* and *Ureaplasma* spp., including *U. parvum* and *U. urealyticum* (Rahman *et al*., 2016). In most cases, *M. hominis* and *U. urealyticum* infections are asymptomatic, and these infections can be associated with an increased risk of adverse pregnancy outcomes, such as miscarriage, stillbirth, and preterm labour (Lee *et al*., 2016). *Mycoplasma hominis*, often reported as a common commensal of the female genital tract has been associated with conditions, such as bacterial vaginosis, cervicitis, endometritis, pelvic inflammatory disease (PID), postpartum septicaemia and pyelonephritis (Rahman *et al*., 2016). *Ureaplasma urealyticum* is considered as the main cause of conditions, such as abortion, bacterial vaginosis (BV), cervicitis, chorioamnionitis, nonchlamydial, non-gonococcal urethritis, preterm birth and preterm delivery (Rahman *et al*., 2016).

Bacterial vaginosis is considered the most common disturbance of the vaginal microflora in which the normally dominant lactobacilli are replaced with increased concentrations of a diverse array of bacteria, which include facultative and anaerobic organisms, such as *Bacteroides* spp., *Gardnerella vaginalis*, genital mycoplasmas and *Mobiluncus* spp. (Gallo *et al*., 2012). Bacterial vaginosis is associated with engagement in sexual activity with new and multiple sex partners (male and female) (Kenyon *et al*., 2013). This condition has been referred to as one of the most prevalent enigmas in the field of medicine and is associated with significant clinical sequelae including increased risk of HIV acquisition, preterm delivery and PID (Kenyon *et al*., 2013; Salah *et al*., 2013).

Although culture is the best method available to monitor the antimicrobial susceptibility profiles of genital mycoplasmas, the development of commercially available diagnostic assays, which are based on liquid broth culture methods, offer a simpler alternative to conventional culture because these methods are easy to perform and to interpret results compared to culture as the results are based on colour changes (D'Inzeo *et al*., 2017). Mycofast Revolution (EliTech Diagnostic, Puteaux, France) and MycoplasmaIST2 (bioMérieux, Marcy l'Etoile, France) are two widely used commercial assays for the detection of *M. hominis* and *U. urealyticum*, and are similar with regards to identification, antimicrobial susceptibility testing and turnaround time. Both assays are based on growth, biochemical identification and antibiotic susceptibility (D'Inzeo *et al*., 2017). Both assays are based on the ability of *Ureaplasma* spp. and *Mycoplasma hominis* to metabolize urea and arginine respectively, releasing ammonia (NH_3). Then NH_3 causes an increase in the pH of the liquid medium and the corresponding colour change of the indicator is used to read the results (D'Inzeo *et al*., 2017). A selective agar medium like A7 *Mycoplasma* agar (bioMérieux, France) can be used to distinguish between *M. hominis* and *Ureaplasma* spp. colonies (Naher and Said, 2013). This medium combines a rich nutritive base containing peptones, horse serum and growth factors (cysteine, arginine, urea) favouring the growth of mycoplasmas (Naher and Said, 2013).

Genital mycoplasma and ureaplasma infections are commonly diagnosed by culture (Gdoura *et al*., 2007). However, given the time-consuming culture, which requires to 2 to 5 days for *Ureaplasma* spp. and *M. hominis* and up to 8 weeks for *M. genitalium*, infectious agents can be detected in less than 8 h by nucleic acid amplification techniques (Gdoura *et al*., 2007). A Multiplex-PCR assay is a technique that is able to detect two or more DNA targets simultaneously in a single amplification reaction (Amirmozafari *et al*., 2009). A Multiplex-PCR assay is able to differentiate between *U. parvum* and *U. urealyticum*, which conventional culture methods are unable to do (Amirmozafari *et al*., 2009).

Bacterial resistance to routinely available antimicrobial agents is a growing and a worldwide problem (Rahman *et al*., 2016). Genital mycoplasmas lack a rigid cell wall and this characteristic renders them innately resistant to antimicrobial agents, such as β-lactam antibiotics and vancomycin, which target the cell wall (Rahman *et al*., 2016). Tetracyclines and quinolones are the antibiotics of choice against genital mycoplasmas; however, macrolides are empirically used to treat pregnant women, as tetracyclines and quinolones are contraindicated in pregnancy (Lee *et al*., 2016). Although macrolides are often the antibiotics of choice for treating these infections, *M. hominis* is intrinsically resistant to the C14 and C15 macrolides (e.g. azithromycin and erythromycin) (Rahman *et al*., 2016). *Ureaplasma* spp. also harbour natural resistance to lincosamides (e.g. clindamycin) (Rahman *et al*., 2016). The resistance observed to macrolides is associated with mutations in the 23S rRNA gene (Rahman *et al*., 2016). The increase in resistance of genital mycoplasmas to antimicrobial agents has prompted the implementation of several ongoing surveillance studies (Rahman *et al*., 2016). It is important to monitor the antimicrobial susceptibility profiles of the mycoplasmas to ensure that the antibiotics used for treatment remain effective. Commercially available assays allow for the rapid detection, identification and determination of antimicrobial susceptibility profiles of genital mycoplasmas. This study detected and identified genital mycoplasmas using conventional culture methods and conventional multiplex-PCR assay as well as their antimicrobial susceptibility profiles in women visiting an antenatal and reproductive biology clinic.

3.3. Methods

3.3.1 Study site and specimen collection

Women attending the antenatal or the reproductive biology clinic at an academic hospital in Pretoria, Gauteng South Africa were approached to participate in this study. The inclusion criteria were: women who are older than 18 years; who visited either the antenatal or the reproductive clinic and who gave written informed consent. Pregnant women at any gestation period were included. Exclusion criteria included women younger than 18 years old or women who did not give informed consent. It was stipulated that all participants should not have taken any antimicrobial agents prior to specimen collection; participants who did not meet this criterium were excluded from the study. The participants were provided with swabs to self-collect the specimens; instructions were included and explained in the informed consent document. Three self-collected Copan swabs (Copan Diagnostics, Inc, Italy) and one BD Eswab (Becton Dickson, Sparks, US) were obtained from each of the particpants visiting the two clinics. Demographic data such as the HIV status, marital status and other relevant information were recorded. All specimens were given unique laboratory numbers to protect the patient's identity and to ensure confidentiality and were processed at the Department of Medical Microbiology. Ethical approval (1/2015) was obtained from the Research Ethics Committee, Faculty of Health Sciences (University of Pretoria) prior to commencement of this study.

The first swab was introduced into the 3 mL mycoplasma broth tube with antimicrobial agents and preservative solution, pH: 6.0 ± 0.1 (UMMt Transport medium) (ELiTech Diagnostic, France). The swab was squeezed against the wall of the tube to recover all the material to be examined. The used swab was discarded into the biohazard box and the tube was transported to the laboratory in the UMMt medium on ice. The inoculated UMMt medium was kept for 56 h at 2ºC to 8ºC or 3 days at -20ºC after two drops of "MYCOPLASMA Stabilizer" were added, if the Mycofast Revolution (ELiTech Diagnostic, Puteaux, France) assay was not performed immediately after collection. The second swab (Copan Diagnostics, Inc, Italy) was used to prepare a smear at the clinic after collection by rolling the swab in a Z pattern onto a glass slide. The prepared smear was then stored in the microscope slides rack and then transported to the laboratory at room temperature

(25ºC±5°C). The third swab (Copan Diagnostics, Inc, Italy) was inoculated into the Mycoplasma R1 vial (3 mL broth) immediately after collection. The inoculated Mycoplasma R1 (3 mL) vials were transported to the laboratory protected from light on ice within 24 h after collection. The inoculated Mycoplasma R1 (3 mL broth in vial) was then stored for 48 h at 2ºC to 8ºC if the test was not performed immediately after collection. The fourth swab was a BD Eswab (Becton Dickson, Sparks, US), which was immediately, after collection, placed in the BD Eswab transport tube with the transport medium. The BD Eswab was transported to the laboratory on ice and was stored at -20ºC and used for the conventional PCR assay.

3.3.2 Specimen processing

The first swab in the UMMt Transport medium (ELiTech Diagnostic, France) was used for detection and enumeration of *Mycoplasma hominis* and *Ureaplasma* species using the Mycofast Revolution (ELiTech Diagnostic, Puteaux**,** France) assay. Slides on which vaginal smears have been made were stored at room temperature (25°C±5°C) until Gram-staining was performed for Nugent scoring. The third swab, which was inoculated into the Mycoplasma R1 vial was used for culturing mycoplasmas on A7 *Mycoplasma* agar plates (bioMérieux, Lyon, France). The content of the Mycoplasma R1 solution was transferred to the Mycoplasma R2 (Lyophilized Urea-Arginine broth) vial upon arrival in the laboratory. This was referred to as the Urea-Arginine LYO 2 broth (Mycoplasma R1+ Mycoplasma R2), which were stored for 48 h at 2ºC to 8ºC if not used immediately. The fourth BD Eswab (Becton Dickson, Sparks, US) was stored at –20°C until DNA extraction was performed.

3.3.3 Culturing of *Ureaplasma* spp. and *Mycoplasma hominis*

Urea-Arginine LYO 2 broth (Mycoplasma R1+ Mycoplasma R2) was incubated (Vacutec, South Africa) for 48 h at 36°C ± 2°C and after that the results were recorded, where the red colour was read as positive and yellow as negative. Before inoculation, the A7 *Mycoplasma* agar plates (bioMérieux, Lyon, France) were brought to room temperature (25ºC±5ºC) to dry the surface of the agar. Starting with the Urea-Arginine LYO 2 broth (Mycoplasma R1+ Mycoplasma R2) containing the specimen under examination, three drops of 10 µL Urea-Arginine LYO 2 broth were dispensed onto the A7 selective *Mycoplasma* agar medium (bioMérieux, Lyon, France) without streaking or the drops touching each other. The inoculated plates were left to dry for 5 min at room temperature (25ºC±5ºC) and placed inside the anaerobic jars. Anaerobic conditions were created by using controlled atmosphere generators called GENbox (bioMérieux, Lyon, France). The plates were incubated (Labcon, California, US) and were observed after 24 h and 48 h of incubation and, depending on growth, the plates were incubated further if necessary for up to 5 days. After incubation, growth was observed using an inverted microscope (American Optical Corporation, Southbridge, US) with the 10x objective. The presence of the following characteristic colonies were recorded: *M. hominis*; round, "fried egg" colonies of 100 to 300 µm and *Ureaplasma* spp; brownish-black "sea urchin" colonies of 10 to 50 µm.

3.3.4. Determination of antimicrobial susceptibility testing using the Mycofast Revolution assay

After collection, a Rayon dry swab (Copan, Brescia, Italy) was used to seed a 3 mL transport (UMMt) medium vial of the Mycofast Revolution assay (EliTech Diagnostic, Puteaux, France). The swabs and the transport media were processed according to the manufacturer's instructions. Briefly, 100 µL of seeded UMMt medium was added to the *M. hominis* (MH) and *U. urealyticum* (UU) wells of the Mycofast Screening Revolution tray with an additional 50 µL of MH supplement (S.Mh) added to the MH well. The wells were covered with two drops of sterile mineral oil and the tray was incubated (Vacutec, South Africa) at 37°C ± 1°C for 24 h. After incubation, the wells were observed for any colour changes. Orange or red colour changes indicated the presence of *M. hominis* and/or *Ureaplasma* spp., whereas yellow wells marked the absence of mycoplasmas. In the case of a positive screening test, the excess UMMt medium that was stored at 2°C to 8°C was used to inoculate the Complement Mycofast Revolution tray (bioMérieux, Lyon, France). Wells 1 to 20 were filled with 100 µL of seeded UMMt medium; wells 6 to 7 were filled with an additional 50 µL of S.Mh and all the wells were covered with two drops of sterile mineral oil. The tray was incubated (Vacutec, UK) at 37°C ± 1°C for 24 h (maximum 48 h in all cases) and after incubation observed for colour changes.

3.3.5 Diagnosis of bacterial vaginosis using Nugent score

The smears were fixed to the slides by heating for 5 s using a Bunsen burner, Gram-stained and used for BV detection by the Nugent scoring system (Nugent *et al.*, 1991). Briefly, Gram staining was performed by flooding the heat-fixed smear with crystal violet (Diagnostic Media Products, NHLS, South Africa) for 1 min. Excess crystal violet (Diagnostic Media Products, NHLS, South Africa) was removed and iodine solution (Diagnostic Media Products, NHLS, South Africa) was added for 1 min to help bind the first dye to the bacterial cell wall components. The third step involved a brief decolourisation with ethanol (Merck, Germany) for ± 30 s. In the final step fixed bacterial cells were counterstained 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 (Madigan and Martinko, 2006).

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 (Nugent *et al*., 1991). 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) (Nugent *et al*., 1991). A 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 (Nugent *et al*., 1991). The integrity of the Nugent scoring results was ensured by having a second analyst to verify the results of the principal investigator by reading the slides.

3.3.6 Genomic DNA extraction of the swabs using a commercial kit

Genomic DNA was isolated from the fourth BD Eswab (Becton Dickson, Sparks, US) with the ZR Fungal/Bacterial DNA kit (Zymo Research, Irvine, US) according to the manufacturer's instructions. Briefly, the swab material was placed in a sterile 2 mL microcentrifuge tube (Axygen, US) and 2 mL 1X phosphate-buffer saline (PBS) (Gibco, Invitrogen, Waltham, US) was added to re-suspend the bacterial cells. The tube was vortexed (VELP Scientifica, Usmate Velate, Italy) for ± 5 min at maximum speed. The ZR BashingBead Lysis Tube (Zymo Research, Irvine, US) was centrifuged (Spectrafuge, Labnet International, Edison, US) 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, US) in a collection tube and centrifuged (Spectrafuge, Labnet international, Edison, US) 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, US) was added to the Zymo-spin IIC column (Zymo Research, Irvine, US) in a new collection tube and centrifuged (Spectrafuge, Labnet International, Edison, US) 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, US) and centrifuged (Spectrafuge, Labnet International, Edison, US) at 10 000 x g for 1 min. The Zymo-spin IIC column (Zymo Research, Irvine, US) was transferred to a clean 1.5 mL micro-centrifuge tube (Axygen, Union city, US) and 100 µL DNA elution buffer (Zymo Research, Irvine, US) was added directly to the column matrix and centrifuged (Spectrafuge, Labnet International, Edison, US) 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 Conventional multiplex PCR assay for detection of mycoplasmas and ureaplasmas

A multiplex PCR assay was performed with primers specific for the highly conserved regions in 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 products sizes can be found in Table 3.2.

The Qiagen multiplex PCR kit (Qiagen, Germany) was used and contains 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 as displayed in Table 3.3 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 100 µM) and TE buffer [10 mM Tris-HC1 (pH 8; Sigma-Aldrich, Germany), 1 mM EDTA (Sigma-Aldrich, Germany)].

Amplification was performed in a G-storm thermocycler (Vacutec, UK) using the following cycling conditions: 1 cycle of 15 min at 95°C, followed by 40, three-step cycles of 94°C for 30 s, 60°C for 90 s and 72°C, followed by a final extension of 10 min at 72°C. Amplified products were visualised for specific fragments under UV light after electrophoresis for 1 h at 100 V on a 1% (m/v) MetaPhor agarose gel (Lonza, US) in 1X TBE buffer [45 mM Tris-borate (Sigma-Aldrich, Germany), 1 mM EDTA (Sigma-Aldrich, Germany)]. A 100 base pair (bp) DNA ladder (Fermentas, Thermo Scientific, US) was included on each gel in order to determine the amplified product sizes.

3.3.8 Data analysis

Microsoft Excel (Microsoft, Redmond, Washington, US) was used for data capturing and statistical analysis. Prevalence of genital mycoplasmas as well as the susceptibility and resistance were determined as percentage. The average, minimum and maximum values were determined for patient demographics.

3.4 Results

Hundred and six female participants who attended either an antenatal (55) or a reproductive biology clinic (51) at an academic hospital in Pretoria, Gauteng South Africa were included in this study. All the participants provided informed consent prior to sample collection. The ages of the participants from the antenatal clinic ranged from 19 to 43 years old with an average of 29 and for those from the reproductive biology clinic the age range was from 20 to 43 years old with an average of 32 (Table 3.9).

The culturing of *Ureaplasma* spp. and *M. hominis* was performed using Urea-Arginine LYO 2 broth (Mycoplasma R1+ Mycoplasma R2) and A7 agar (Table 3.4). In the antenatal clinic, 81% (45/55) positive Urea-Arginine LYO 2 broth cultures were detected and 38% (11/29) positive cultures using A7 agar media. In the reproductive clinic, 59% (30/51) positive Urea-Arginine LYO 2 broth cultures and 18% (7/40) positive culturse using A7 agar media were detected.

Bacterial vaginosis was diagnosed using the Nugent scoring system and it showed that 24% (13/51) of the participants had intermediate vaginal flora and 20% (11/55) of the participants were BV positive in the antenatal clinic (Table 3.7). In the reproductive biology clinic, 22% (11/51) of the participants had intermediate vaginal microflora and 20% (10/51) of the participants were BV positive (Table 3.7). All 11 specimens (100%) with a high Nugent score (BV positive) were positive for at least one genital mycoplasma species in the antenatal clinic. *Ureaplasma* spp. was present in 10 (91%) of the BV positive specimens, whereas *M. hominis* was present in seven (64%) of the BV positive specimens. In the reproductive biology clinic seven of the 11 specimens (64%) with a high Nugent score (BV positive) were positive for at least one genital mycoplasma. *Ureaplasma* spp. were present in six (55%) of BV positive specimens, whereas *M. hominis* was present in four (36%) of the BV positive specimens.

The Mycofast Revolution assay was performed to detect and determine antimicrobial susceptibility of genital mycoplasmas in the antenatal and reproductive biology clinics with red wells indicating positive reactions and yellow wells indicating negative reactions (Figure 3.1). Genital mycoplasmas were detected in 67% (37/55) of the participants specimens attending the antenatal clinic. Sixty-four percent (35/55) of cultures were positive for *Ureaplasma* spp., and 27% (15/55) of the cultures were positive for *M. hominis*. Mixed strains (*M. hominis* and *Ureaplasma* spp.) were present in 24% (13/55) of specimens. *Mycoplasma hominis* alone was present in 4% (2/55), while *Ureaplasma* spp. alone were present in 38% (21/55). In the samples from participants attending the reproductive biology clinic, genital mycoplasmas were detected in 57% (29/51) of samples. Fifty-three percent (27/51) of cultures were positive for *Ureaplasma* spp., and 18% (9/51) of the cultures were positive for *M. hominis*. Mixed strains (*M. hominis* and *Ureaplasma* spp.) were present in 14% (7/51) of cultures. *Ureaplasma* spp. alone were present in 37% (19/51), while *M. hominis* alone was present in 4% (2/51).

In the participant specimens from the antenatal clinic, susceptibility rates of the *Ureaplasma* spp. of 89% (31/35) and 80% (28/35) were detected for levofloxacin and moxifloxacin respectively. Thirteen percent (2/15) of *Ureaplasm*a spp. were susceptible to clindamycin, while 74% (26/35) of *Ureaplasma* spp. were susceptible to erythromycin. Twenty-six percent (9/35) of *Ureaplasma* spp. were resistant to erythromycin. Resistance to tetracycline was detected in 51% (18/35) of *Ureaplasm*a spp.. *Mycoplasma hominis* susceptibility rates of 53% (8/15) and 60% (9/15) were detected for levofloxacin and moxifloxacin respectively. Eighty-seven percent (13/15) of *M. hominis* were resistant to clindamycin. Tetracycline resistance was detected in 51% (18/35) of the *M. hominis* (Table 3.5).

Susceptibility rates for levofloxacin and moxifloxacin in the *Ureaplasma* spp. in samples obtained from the reproductive biology clinic were 82% (22/27) and 93% (25/27). Forty-one percent (11/27) of the *Ureaplasma* isolates were resistant to erythromycin. Resistance to tetracycline was detected in 52% (14/27) of these isolates. *Mycoplasma hominis* susceptibility rates of 67% (6/9) and 33% (3/9) were detected for levofloxacin and moxifloxacin respectively. Seventy-eight percent (7/9) of *M. hominis* were resistant to clindamycin and 22% (2/9) of *Ureaplasma* isolates were susceptible to clindamycin. *Mycoplasma hominis* resistance to tetracycline was detected in 56% (5/9) (Table 3.6).

A conventional multiplex PCR assay with gel electrophoresis (1%) was used for the detection of the different genital mycoplasmas (Figure 3.2). *Mycoplasma hominis, M. genitalium*, *U. parvum* and *U. urealyticum* were detected in 5% (3/55), 2% (1/55), 16% (9/55) and 5% (3/55) respectively at an antenatal clinic. In the reproductive biology clinic 4% (2/51), 22% (11/51) and 2% (1/51) of *M. hominis*, *U. parvum* and *U. urealyticum* were detected respectively. *Mycoplasma genitalium* was detected only in an antenatal clinic in 2% (1/51) of the participant specimens analysed. The overall prevalence of *M. genitalium*, *M. hominis*, *U. parvum* and *U. urealyticum* in both clinics combined was 1% (1/106), 5% (5/106), 19% (20/106) and 4% (4/106).

3.5 Discussion

In resource constrained countries, it has been shown that the syndromic treatment of STIs is appropriate for high-risk populations and symptomatic patients, whereas in asymptomatic individuals, especially women, risk scores and simple laboratory tests may be required to boost the syndromic management algorithm's sensitivity (Martin *et al*., 2006; Díaz *et al*., 2013). The gold standard for diagnosis of genital mycoplasmas infections is culture especially in low to moderate test volume laboratories and it is still considered the most cost effective strategy (Stellrecht *et al*., 2004; D'Inzeo *et al*., 2017). Nucleic acid amplification-based tests have higher specificity and sensitivity than culture and are the only means for detection of *M. genitalium* and for discriminating between the *Ureaplasma* spp. (Stellrecht *et al*., 2004; D'Inzeo *et al*, 2017). It is recommended that cultivation systems include a solid agar medium as well as supplemental broths for optimal isolation of genital mycoplasmas from clinical specimens (Fiacco *et al*., 1984*)*. In this study, the cultivation of genital mycoplasmas was performed using the Urea-Arginine LYO 2 broth (Mycoplasma R1+ Mycoplasma R2), A7 agar and the Mycofast Revolution assay, which can also determine antimicrobial susceptibility.

The Urea-Arginine LYO 2 broth (Mycoplasma R1+ Mycoplasma R2) detected a high positive rate, which cannot be a true reflection of genital mycoplasma positivity. This is because other bacteria can also grow in the broth and cause the colour change. Genital mycoplasmas were poorly recovered from A7 agar medium in this study. Low recovery rates of genital mycoplasmas from clinical specimens have been reported previously (Waites *et al*., 2009; Govender, 2010). Genital mycoplasmas are difficult to culture, are susceptible to environmental conditions, such as toxic metabolites and fluctuations in temperature, and are prone to contamination (Xiao, 2010). The culture positivity rate of genital mycoplasmas in our study in the antenatal clinic is in agreement with findings reported by Stellrecht *et al.* (2004) of 25% (21/85) from a fertility clinic between November 1998 and November 1999 in the US.

Bacterial vaginosis is mostly not symptomatic in the genital tract of women, which may happen in half of the cases and its prevalence varies in diferent communities (Mengistie *et al*., 2014). The prevalence of BV varies from 17.7% in pregnant women to 40% in sex workers in Israel and Tanzania (Dan *et al*., 2003; Riedner, 2003). It has been suggested that the socio-economic status as well as health and hygiene conditions have important roles in the higher incidence of BV in some populations (Dan *et al.,* 2003).

In our study, the positivity rate of BV was 20% (11/55) and 20% (10/51) of the participants from the antenatal and reproductive clinic respectively. Our results are consistent with the results reported by Sabour *et al*. (2018) among Iranian pregnant and non-pregnant women who reported overall 18.9% BV positives in both group of women. In their study, the prevalence of BV among pregnant women was 16.5%, which is slightly less than our finding in the antenatal clinic (Sabour *et al*., 2018). Similar results to our study have been reported in the study conducted by Lata *et al*., 2010 which included 200 women attending an antenatal clinic (ANC) of a tertiary hospital in North India who reported 20.5%. Due to some important adverse effects of BV in pregnant women such as preterm premature rupture of membranes, preterm labor, abortion and postpartum infections, this syndrome is an important gynaecologic problem around the world (Sabour *et al*., 2018). Therefore, pregnant women in early stages of pregnancy need to be screened for BV to prevent abortion and preterm delivery (Sabour *et al*., 2018). Despite the high incidence of BV, especially among asymptomatic women, clinicians as well as women may ignore this syndrome (Sweet, 2000).

The positivity rates of *M. hominis* and *Ureaplasma* spp. reported in this study using the Mycofast Revolution assay was 67% (37/55) in the samples obtained from participants attending the antenatal clinic and 57% (29/51) in the reproductive clinic. These findings are in agreement with that reported by Redelinghuys *et al.* (2014) who tested pregnant women attending the antenatal clinic at the tertiary academic hospital in Pretoria, Gauteng South Africa and reported a prevalence of 71% (35/49). Rahman *et al*. (2016) conducted a study among a group of Egyptian pregnant women attending an antenatal care clinic using Mycoplasma IES kit and out of the 50 vaginal swabs, 56% (28/50) of women were positive for either *M. hominis*, *U. urealyticum* or both. The positivity rate of 57% (29/51) obtained from the participants attending the reproductive clinic was in agreement with the results of the study conducted by Rahman *et al.* (2016) among a group of Egyptian pregnant women attending an antenatal care clinic using Mycoplasma IES kit. Our results are similar to that reported by Zhu *et al.* (2012), who reported an overall 62.16% positive incidence of genital mycoplasmas in 3306 female patients with genital tract infections admitted to the Gynaecological Department of Changzhou Fourth Hospital Soochow University and Changzhou Tumor Hospital, in China between January 2005 and December 2009.

The prevalence of genital mycoplasmas varied among different studies in different countries. Bayraktar *et al.* (2010) who conducted a study in 100 pregnant women of whom 29% (29/100) were positive for *M. hominis* and/or *U. urealyticum* reported a lower prevalence rate. In a study conducted from January 2004 to June 2011 by De Francesco *et al.* (2013) the endocervical, urethral, or vaginal swabs obtained from the urogenital tracts of 9 956 adults (aged between 18 and 43 years) who visited Brescia's main hospital (Spedali Civili), were tested for urogenital mycoplasmas. A genital mycoplasma prevalence of 18.6% (1856/9956) was observed, of these, 1652 were positive for *U. urealyticum* (89%), 21 were positive for *M. hominis* (1.1%), and 183 were positive for both (9.8%). Wang *et al.* (2014) who conducted a study on female outpatients treated for genital infections at a Chinese hospital from January 1, 2009 to December 31, 2013 also reported a lower percentage with a total positivity rate of 33.9% for 6051 female outpatients.

Ureaplasma spp. were the most commonly identified species and contributed to the high prevalence of genital mycoplasmas found in this study. Several studies also reported higher rates of *Ureaplasma* species compared to *M. hominis* (Zhu *et al.* 2012; Redelinghuys *et al.*, 2014). Redelinghuys *et al.* (2014) reported a higher rate of infection with 76% (73/96) of specimens containing *Ureaplasma* spp, which is in agreement with the rate of *Ureaplasma* spp. in our study in both clinics. Zhu *et al.* (2012) also reported *Ureaplasma* spp. as the dominant species with a *U. urealyticum* positivity of 46.52%, and *M. hominis* positivity of 1.71%.

In this study *Ureaplasma* spp. alone were detected in higher rates followed by mixed infections (both *Ureaplasma* spp. and *M. hominis*) and *M. hominis* single infection as the lowest detected species. Our results are in agreement with similar studies, which reported the rates of *Ureaplasma* spp. alone ranging from 27% to 68.9%, mixed infections ranging from

6% to 26.7% and *M. hominis* single infection ranging from 1.71% to 5% (Bayraktar *et al*., 2010; Zhu *et al*., 2012; Díaz *et al*., 2013;).

Resistance of *Ureaplasma* species to erythromycin in the antenatal (26%) and reproductive biology clinic (41%) was lower compared to that reported by other studies (80% to 83%) (Kechagia *et al*., 2008; Redelinghuys *et al*., 2014). The *Ureaplasma* spp. were less resistant to erythromycin in this study for both clinics compared to a similar study conducted by Tetracycline resistance for *Ureaplasma* spp. reported in this study was higher compared to the findings reported by Díaz *et al*. (2013) who conducted a study in women with vaginal discharge through analysis of laboratory data from vaginal smears from 255 patients referred to the Municipal Hygiene and Epidemiology Center in Güines, Mayabeque Province, Cuba, which showed 31.2% resistance.

Tetracycline resistance for *M. hominis* reported in this study is similar to the findings reported by Díaz *et al*. 2013 who reported tetracycline resistance of 58%. According to Rahman *et al*. (2016) the wide range of variability in the antimicrobial susceptibility patterns can be attributed to the different antimicrobial-use policies, which lead to the emergence of resistance to one or other antimicrobial group. Other variables contributing to this might include the population studied, the study period, or the kits used for specimen processing and analyses (Rahman *et al*., 2016). In this study the participants included women visiting either an antenatal and reproductive biology clinic In other studies, Mycoplasma IST-2 detection and antimicrobial susceptibility testing was used as compared to our study where the Mycofast revolution kit was used (Bayraktar *et al*., 2010; Lee *et al*., 2017). The laboratory performance of Mycoplasma IES (IES), the Mycofast Revolution (REV) and Mycoplasma IST 2 (IST2) compared to A7 agar plates for the detection of *U. urealyticum* and *M. hominis* in clinical samples was assessed by D'Inzeo *et al*. (2017). In their findings, the Mycoplasma IES kit was found to be equivalent or superior compared to other commercial culture-based assays for a rapid and accurate identification of *U. urealyticum* and *M. hominis* and detection of resistance (D'Inzeo *et al*., 2017).

The conventional multiplex PCR assay was used for the detection of the different genital mycoplasmas. In this study the conventional multiplex PCR assay detected lower positive samples as compared to the Mycofast Revolution assay. These results higlight that there were technical problems when analysing the samples using the conventional multiplex PCR assay. Some studies reported culture having low sensitivity when compared to PCR assays (Cao *et* *al*., 2007; Zeighami *et al*., 2007). Redelinghuys *et al.* (2014) conducted a study on self-collected swabs obtained from pregnant women attending the antenatal clinic of a tertiary academic hospital in Pretoria, South Africa from October 2012 to November 2012. In the study, the authors reported higher positivity rates of genital mycoplasmas with multiplex PCR as compared to Mycofast Revolution assay [89.8% (44/49) and 71.4% (35/49) respectively].

The main limitation of this study was the sample size, which does not include a representative population sample and the technical problems experienced with theconventional multiplex PCR assay. Another limitation was the difficulty experienced in the culturing of genital mycoplasmas on A7 agar, which is prone to contamination and resulted in lower positivity rates. Further research is needed in Pretoria, Gauteng South Africa to see whether these results are representative of the larger population; such research will require a larger sample size and full access to clinical, epidemiological and microbiological variables.

The establishment of common guidelines for the treatment of genital mycoplasma infections is complex and effective treatment depends on the antimicrobial susceptibility patterns of genital mycoplasmas in a specific region (Redelinghuys *et al*., 2014). Monitoring of antimicrobial susceptibility patterns of genital mycoplasmas may assist with optimising treatment guidelines and overall improve therapeutic outcomes (Redelinghuys *et al*., 2014). The Mycofast Revolution assay is an easy and effective way of evaluating the susceptibility of genital mycoplasmas to commonly used or potential antimicrobial agents (Redelinghuys *et al*., 2013).

3.6 Conclusion

This study has shown that genital mycoplasma infection rates were higher among pregnant women. Routine screening for the presence of genital mycoplasmas is recommended to prevent complications in pregnant women, but also to improve the foetal and neonatal well-being. The Mycofast Revolution assay could be considered as a cost-effective alternative to conventional culture methods for the rapid detection of genital mycoplasmas.

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Table 3.1: Nugent scoring system (0 to 10) for Gram-stained vaginal smears

Score[1]	***Lactobacillus* spp**	***Gardnerella vaginalis* and *Bacteroides* spp morphotypes**	***Mobiluncus***
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

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

Table 3.3: Reaction setup and components for conventional multiplex PCR for genital mycoplasmas detection

Component	Volume/reaction	Final concentration
Reaction mix		
▪ 2x Qiagen Multiplex PCR Master Mix	25 µl	1x
▪ 10x primer mix, 2 µM each primer	5 µl	0.2 µM
▪ RNase-free water	15 µl	-
Template DNA	5 µl	≤ 1 µg DNA/50 µl
Total volume	**50 µl**	

Table 3.4: Culture results using Urea-Arginine LYO 2 broth and A7 agar for two clinics

Culture	**clinics**	
	Antenatal (N=55)	**Reproductive biology(N=51)**
A7 agar	37.9% (11/29).	18% (7/40)

Table 3.5: (A) Antenatal clinic distribution (%) of *Ureaplasma* species and *M. hominis* at different breakpoints of antimicrobial agents

Pathogens	**Levofloxacin**		**Moxifloxacin**		**Erythromycin**		**Clindamycin**		**Tetracycline**	
	resistance	**susceptible**	**resistance**	**susceptible**	**resistance**	**susceptible**	**resistance**	**susceptible**	**resistance**	**susceptible**
***Ureaplasma* species (n = 35)**	12% (4/35)	89% (31/35)	20% (7/35)	80% (28/35)	26% (9/35)	74% (26/35)	–	–	51% (18/35)	49% (17/35)
***M. hominis* (n = 15)**	47% (7/15)	53% (8/15)	40% (6/15)	60% (9/15)	–	–	87% (13/15)	13% (2/15)	53% (8/15)	47% (7/15)

Table 3.5: (B) The distribution (%) of *Ureaplasma* spp. and *M. hominis* at different breakpoints of antimicrobial agents (n = 35) in the antenatal clinic

	Levofloxacin			Moxifloxacin		Erythromycin		Clindamycin		Tetracycline			
	1[1]	2	4	0.25	2	8	16	0.25	0.5	1	2	4	8
*Ureaplasma*species (n = 22)	59	18	9.1	68	23	50	14	100	82	41	41	36	31
*Ureaplasma*species and *M. hominis* (n = 13)	85	54	15	46	23	62	54	100	92	77	69	69	61

Table 3.6: (A) Reproductive biology clinic distribution (%) of *Ureaplasma* species and *M. hominis* at different breakpoints of antimicrobial agents

Pathogens	Levofloxacin		Moxifloxacin		Erythromycin		Clindamycin		Tetracycline	
	resistance	susceptible	resistance	susceptible	resistance	susceptible	resistance	susceptible	resistance	susceptible
Ureaplasma species (n = 27)	82% (22/27)	19% (5/27)	7% (2/27)	93% (25/27)	41% (11/27)	59% (16/27)			52% (14/27)	48% (13/27)
M. hominis (n = 9)	33% (3/9)	67% (6/9)	67% (6/9)	33% (3/9)	–	–	78% (7/9)	22% (2/9)	56% (5/9)	22% (2/9)

Table 3.6: (B) The distribution (%) of *Ureaplasma* spp. and *M. hominis* at different breakpoints of antimicrobial agents (n = 29) in the reproductive biology clinic

	Levofloxacin			Moxifloxacin		Erythromycin		Clindamycin		Tetracycline			
	1[1]	2	4	0.25	2	8	16	0.25	0.5	1	2	4	8
*Ureaplasma*species (n = 22)	45	15	5	65	5	40	30	90	75	60	40	40	25
*Ureaplasma*species and *M. hominis* (n = 7)	43	29	14	86	14	86	86	100	100	86	85	86	71

Table 3.7: Results for bacterial vaginosis obtained from an antenatal and reproductive biology clinic

Bacterial vaginosis	Clinic name		Overall n=106
	Antenatal N=55	Reproductive biology N=51	
Negative	31 (56%)	30 (59%)	61 (58%)
Intermediate	13 (24%)	11 (22%)	24 (23%)
Positive	11 (20%)	10 (20%)	21 (20%)

Table 3.8: Conventional multiplex PCR assay for detection of mycoplasmas and ureaplasmas (*M. hominis*, *M. genitalium*, *U. urealyticum* and *U. parvum* detection)

Pathogen and gene of interest	Antenatal clinic N=55	Reproductive biology clinic N=51
M. genitalium 140-kDa adhesion protein gene (282 bp)	2% (1/55)	0
M. hominis 16S rRNA (334 bp)	5% (3/55)	4% (2/51)
U. Parvum MB antigen gene (403bp)	16% (9/55)	22% (11/51)
U. urealyticum MB antigen gene *(448 bp)*	5% (3/55)	5% (3/51)

Table 3.9: Summary of the demographics of the patients obtained from an antenatal and reproductive biology clinic

Variables	Clinic name	
	Antenatal N=55	**Reproductive biology N=51**
Age	29.36 (19-43)	32 (20-43)
Ethnicity		
African	44 (80%)	41 (80%)
White	10 (18%)	6 (12%)
Caucasian	1 (2%)	4 (8%)
Marital status		
Married	22 (40%)	43 (84%)-
Single	33 (60%)	8 (16%)
Divorced	0	0
Gestational weeks	28.13 (5-39)	-
HIV status		
Positive	11 (20%)	7 (14%)
Negative	44 (80%)	44 (86%)
Tampon use		
Yes	3 (5%)	5 (10%)
No	52 (95%)	46 (90%)
New sexual partners (last 6 months)		
Yes	1 (2%)	1 (2%)
No	54 (98%)	50 (98%)
Douching		
Yes	4 (7%)	8 (16%)-
No	51 (93%)	43 (84%)
Condom use		
Always	0	2(4%)
Yes	18 (33%)	4 (8%)
No	37 (67%)	43 (84%)
Alcohol consumption		
Yes	1 (2%)	13 (25%)
No	54 (98%)	38 (75%)
Vaginal sex frequency	1.4 (0-5)	2.3 (0-4)

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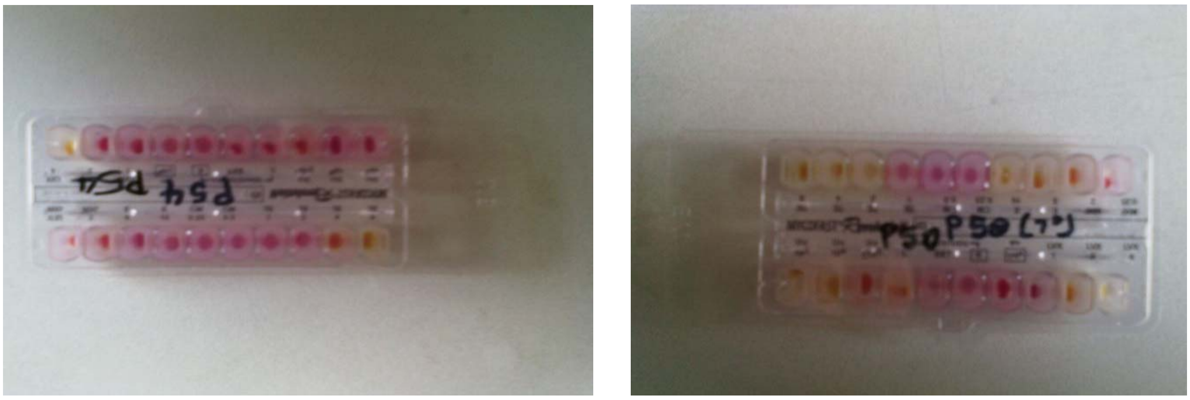

Figure 3.1: Appearance of Mycofast revolution tray with positive culture after 24 hours. Red wells indicate positive results and yellow wells indicate negative results

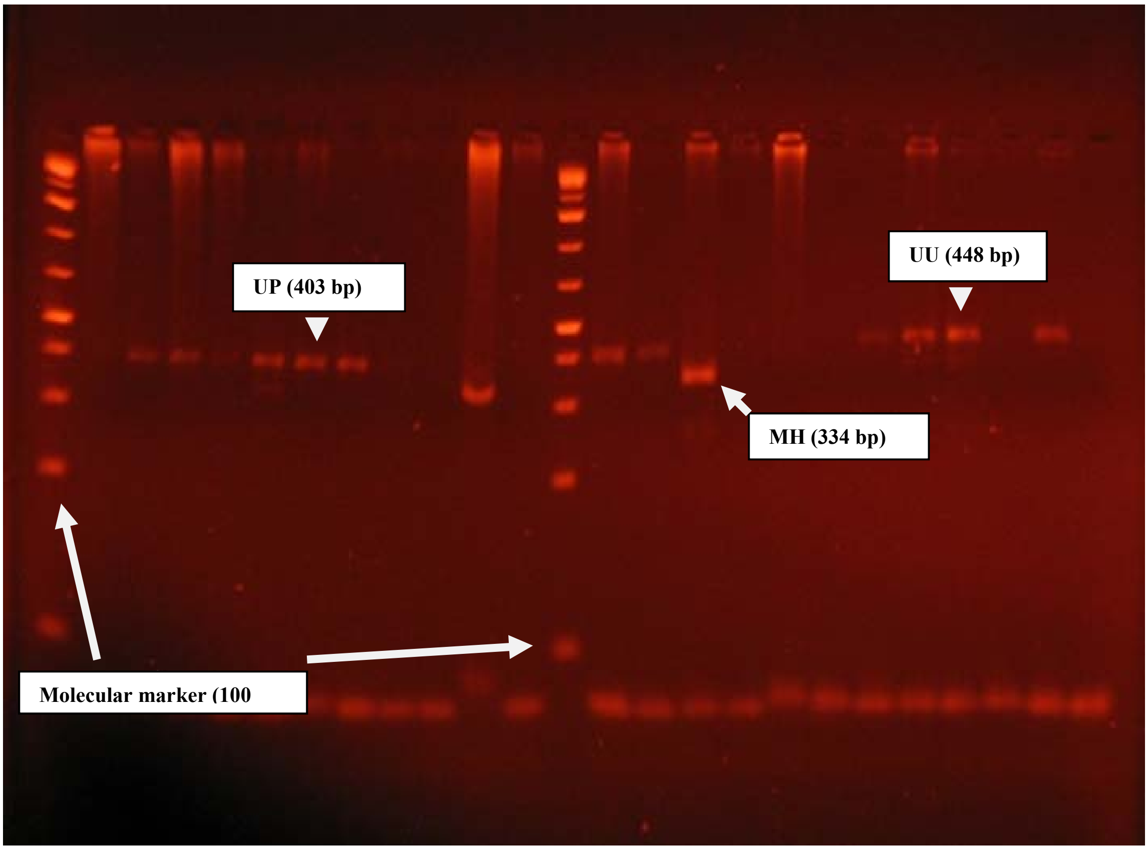

Figure 3.2 Gel electrophoresis analysis of an mPCR assay for the detection of genital mycoplasmas on a 1% (m/v) MetaPhor agarose gel (Lonza, USA). *Mycoplasma genitalium* 140-kDa adhesion protein gene at 282 bp, *Mycoplasma hominis* 16S rRNA at 334 bp, *Ureaplasma parvum* and *Ureaplasma urealyticum* MB antigen gene at 403 and 448 bp respectively

CHAPTER 4

DETECTION OF SEXUALLY TRANSMITTED INFECTIONS IN WOMEN VISITING AN ANTENATAL AND REPRODUCTIVE BIOLOGY CLINIC OF A TERTIARY HOSPITAL, SOUTH AFRICA

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

4.1 Abstract

Introduction

Worldwide, sexually transmitted infections (STIs) represent a major global health problem with 340 million cases every year. These infections can be transmitted between humans by means of sexual activity including vaginal intercourse, oral sex and anal sex. Major STI pathogens include the genital mycoplasmas (*Mycoplasma genitalium*, *Mycoplasma hominis*, *Ureaplasma parvum* and *Ureaplasma urealyticum*) and other pathogens such as *Chlamydia trachomatis, Neisseria gonorrhoeae* and *Trichomonas vaginalis.* Sexually transmitted infections are associated with a wide spectrum of disease presentations, such as urethritis and epididymitis in men and cervicitis in women. Currently in South Africa especially in the Gauteng region, there is limited information available on the prevalence of STIs in women visiting antenatal and reproductive biology clinics. The aim of this study was to determine the prevalence of genital mycoplasmas and bacterial co-infections in women visiting antenatal and reproductive biology clinics.

Methods:

Female participants who attended either an antenatal or a reproductive biology clinic at an academic hospital in Pretoria, Gauteng South Africa were asked to participate in this study. As part of a bigger study four self-collected vaginal swabs were obtained from each of the participants, visiting the clinics after written informed consent was obtained. One of the swabs was used for genomic DNA extraction using a commercial kit and analysed using the Anyplex II STI-7 (Seegene, Seoul, Korea) multiplex real-time PCR assay for detecting seven STIs (*C. trachomatis*, *M. genitalium*, *M. hominis*, *N. gonorrhoeae, T. vaginalis*, *U. parvum* and *U. urealyticum*) simultaneously.

Results:

Swabs were obtained from 106 participants visiting either an antenatal clinic (55) or a reproductive clinic (51) at an academic hospital in Pretoria, Gauteng, South Africa. The Anyplex II STI-7 detected 64.1% (68/106) of *U. parvum*, 28.3% (30/106) of *M. hominis*, 24.5% (26/106) of *U. urealyticum*, 11.3% (12/106) of *T. vaginalis,* 5.7% (6/106) of *C. trachomatis,* 2% (2/106) of *M. genitalium* and 0.9% (1/106) of *N. gonorrhoeae. Ureaplasma parvum* was the most commonly identified pathogen.

Conclusion:

This study confirms the high prevalence of *U. parvum* among the studied women visiting either an antenatal or a reproductive biology clinic. Routine testing of STIs is encouraged for the identification and management of infections especially in pregnant women as this can lead to severe reproductive complications and adverse pregnancy outcomes.

4.2 Introduction

Sexually transmitted infections (STIs) continue to be a significant public health problem with an increased burden in women of reproductive age (Abbai *et al*., 2013). Sexually transmitted infections are caused by pathogens that can be acquired and transmitted through sexual activity (Shim, 2011). Major STI pathogens include genital mycoplasmas (*M. genitalium*, *M. hominis*, *U. parvum* and *U. urealyticum*) and other pathogens such as *T. vaginalis*, *C. trachomatis* and *N. gonorrhoeae* (Avolio *et al*., 2016; Vică *et al*., 2016). *Mycoplasma hominis* and *U. urealyticum* are most commonly found in the genital tract of women who are sexually active (Bayraktar *et al*., 2010). The colonisation values for *M. hominis* range between 20% and 30% and for *U. urealyticum* it ranges between 60% and 80% (Bayraktar *et al*., 2010). *Mycoplasma genitalium* was first isolated from men who suffered from urethritis (Waites *et al.,* 2012). *Mycoplasma genitalium* is now considered an established cause of male non-gonococcal urethritis (NGU) and female cervicitis (Waites *et al*., 2012; Hamasuna, 2013).

Fertility in both men and women may be adversely affected due to the effect of infections caused by ureaplasmas and mycoplasmas (La Vignera *et al*., 2011). Studies generally agree that the prevalence of ureaplasmas and mycoplasmas is higher among the male partners in infertile couples than in fertile males (Lee *et al*., 2013). Several studies have found higher detection rates for ureaplasmas and mycoplasmas in the lower genital tract and endometrium of infertile women than in fertile women; supporting the association of the two microbes with female infertility (Stray-Pedersen *et al*., 1978; Cassell *et al*., 1983; Lee *et al*., 2013). *Ureaplasma* infection can lead to disturbances in spermatogenesis, sperm function, transport and penetration as well as the death of spermatozoa (Borovkova *et al*., 2011). *Ureaplasma* spp. have been associated with unexplained infertility in women, reduced pregnancy rates after *in vitro* fertilization and endometritis (Borovkova *et al*., 2011). Among the ureaplasmas, *U. parvum* is the most frequently found species in the genital tract of infertile couples (Borovkova *et al*., 2011).

Several commercially available real-time PCR assays enable the simultaneously detection of STI pathogens (Berçot *et al.,* 2015). The Anyplex II STI-7 kit (STI-7, Seegene, Seoul, 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 can simultaneously detect seven microorganisms involved in STIs (Berçot *et al.,* 2015).

The prevalence of genital mycoplasmas infections over the last decade has been increasing globally, which can be in part due to the increase in mycoplasma antibiotic resistance (Rivera *et al*., 2001; Taylor-Robinson *et al.*, 2012). Currently in South Africa, especially in the Gauteng region, there is limited information available on the prevalence of mycoplasmas in women visiting either an antenatal or reproductive biology clinic. Simultaneous detection of genital mycoplasmas and other STIs, such as *C. trachomatis, N. gonorrhoeae* and *T. vaginalis* is vital as these pathogens are involved in early pregnancy loss, stillbirth, preterm birth, and neonatal morbidity, as well as male infertility. It is important to detect these pathogens and intervene early enough to prevent complications and adverse outcomes in pregnancies in a high human immunodeficiency virus (HIV) -prevalence country, such as South Africa. In pregnant women, this might minimise the risk for complications at a later stage, which may reduce the rates of neonatal morbidity and mortality. The purpose of this study was to detect and identify seven STI pathogens simultaneously using real time PCR assay.

4.3 Methods

4.3.1 Study site and specimen collection

This was an investigative study that included women attending an antenatal or a reproductive biology clinic at an academic hospital in Pretoria, Gauteng South Africa. Patients older than 18 years were included in the study. All women who participated gave written informed consent prior to commencement. Ethical approval was obtained from the Student Ethics Committee of the Faculty of Health Sciences, University of Pretoria (protocol number 1/2015) and preceded experimental work. Experimental work was conducted at the Department of Medical Microbiology, University of Pretoria.

A self-collected vaginal BD Eswab (Becton Dickson, Sparks, US) was obtained from women who attended an antenatal (55) and a reproductive biology clinic (51) and were tested for the presence of seven microorganisms that can cause STIs. The BD Eswab was transported to the laboratory and stored at –20ºC until DNA extraction and subsequent molecular detection was performed using the AnyplexTM II STI-7 detection kit (Seegene, Seoul, Korea)..

4.3.2 Genomic DNA extraction of the swabs using a commercial kit

Genomic DNA was isolated from the collected vaginal swab (BD Eswab) using the ZR Fungal/Bacterial DNA kit (Zymo Research, Irvine, US) according to the manufacturer's instructions. A more detailed description can be found in chapter 3, section 3.3.6. After the extraction process, the eluted DNA was stored at -20°C until analysis.

4.3.3 Multiplex real-time PCR assay detection of mycoplasmas, ureaplasmas and co-infections

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 AnyplexTM 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 TriisopropylsilylOxyMethy (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 to 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 results were analysed as either positive or negative, but not quantified.

4.3.4 Data analysis

Microsoft Excel sheets (Microsoft, Redmond, US) was used for data capturing and statistical analysis. Prevalence of genital mycoplasmas and other STIs were determined as percentages. The average, minimum and maximum values were determined for patient demographics.

4.4 Results

Hundred-and-six women who attended either an antenatal (55) or a reproductive biology clinic (51) at a tertiary hospital in Pretoria, Gauteng, South Africa agreed to particpate in this study. The age range of the participants visiting the antenatal clinic was from 19 to 43 years old with an average of 29, while for those attending the reproductive biology clinic the age range was from 20 to 43 years old with an average of 32 (Table 4.1).

The Anyplex II STI-7 real-time PCR assay was used to detect seven STI pathogens from the obtained swab samples. The following results were obtained: 64.1% (68/106) of *U. parvum*, 28.3% (30/106) of *M. hominis*, 24.5% (26/106) of *U. urealyticum*, 11.3% (12/106) of *T. vaginalis,* 5.7% (6/106) of *C. trachomatis,* 2% (2/106) *M. genitalium* and 0.9% (1/106) of *N. gonorrhoeae* (Table 4.2). Mixed infections of *M. hominis* and *Ureaplasma* spp., which refers to having both species in one sample, were present in 25% (27/106) of the samples. *Mycoplasma hominis* alone was detected in 3% (3/106) of samples.

In the samples obtained from participants visiting the antenatal clinic, the Anyplex II STI-7 detected 73% (40/55) *U. parvum,* 36% (20/55) *M. hominis,* 33% (18/55) *U. urealyticum,* 22% (12/55) *T. vaginalis*, 9% (5/55) *C. trachomatis,* 4% (2/55) *M. genitalium* and 2% (1/55) *N. gonorrhoeae*. Seven percent (4/55) of samples were positive for only *Ureaplasma* spp. and 2% (1/55) of the samples were positive for only *M. hominis*. *Ureaplasma parvum* alone was detected in 27% (15/55) of the swab samples. Mixed infections of *M. hominis* and *Ureaplasma* spp. were present in 35% (19/55) of the samples.

In the reproductive biology clinic 51 samples were tested using the real-time PCR assay and the results showed 55% (28/51) *U. parvum,* 20% (10/51) *M. hominis*, 16% (8/51) *U. urealyticum*, and 2% (1/51) *C. trachomatis* (Table 4.1). *Neisseria gonorrhoeae* and *M. genitalium* and *T. vaginalis*, were not detected in any of these participants swab samples. Four percent (2/51) of the samples were positive for *Ureaplasma* spp. and 2% (1/51) of the samples were positive for only *M. hominis*. *Ureaplasma parvum* alone was detected in 37% (19/51). Mixed infections of *M. hominis* with *Ureaplsama* spp. were present in 15.6% (8/51) of the samples (Table 4.2).

4.5 Discussion

Worldwide, sexually transmitted infections (STIs) represent a major global health problem with 340 million of cases every year (Del Prete *et al.*, 2017). Risk factors that influence the increased rate of STIs amongst populations of developing countries have been identified as a combination of behavioural and social factors, demographics and economics (Stoneburner and Low-Beer, 2004). Risk factors that influence the increased rate of STIs amongst populations of developing countries have been identified as urban migration with the accompanying socio-cultural changes, increase in levels of prostitution due to economic hardship, multiple and concurrent sexual partnerships, lack of access to effective and affordable STI services and increased antimicrobial resistance (Mayaud and Mabey, 2004).

The seven STI pathogens detected using the commercially available real-time PCR assay, Anyplex II STI 7 were *C. trachomatis, M. genitalium, M. hominis, N. gonorrhoeae, T. vaginalis, U. parvum* and *U. urealyticum*. *Ureaplasma parvum* was the most commonly identified species and contributed to the high prevalence of genital mycoplasmas found in this study. Some studies reported *U. parvum* to be the most prevalent genital mycoplasmas in reproductive age women with prevalence rates of 17% to 64% (Povlsen *et al*., 2002; Kataoka *et al*., 2006; De Francesco *et al*., 2009; Del Prete *et al*., 2017).

The use of the Anyplex II STI-7 real-time PCR assay in this study assisted to distinguish between the species, *U. parvum* and *U. urealyticum*. Previously, because of the absence of real time PCR techniques, many reports had suggested the role of *Ureaplasma* spp. without discriminating between *U. urealyticum* and *U. parvum* (Del Prete *et al*., 2017). There are contradictory reports as to which *Ureaplasma* spp. can be regarded as 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 aetiological role in both genital infections and male infertility (Andrade-Rocha, 2003). More evidence has become available reporting on the damage caused by *U. urealyticum* to the development and vitality of human embryos (Montagut *et al.,* 1991; Reichart, *et al*., 2000). 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).

In our study, we have also investigated the presence of the mixed infections of *M. hominis* and *U. urealyticum* / *U. parvum*, which refers to having both species positive in one sample. Redelinghuys *et al.* (2014) who conducted a study which included pregnant women attending the antenatal and maternal and foetal unit (MAFU) clinics of a academic hospital in Pretoria, South Africa, from October 2012 to January 2013 reported a higher rate of mixed infections for *M. hominis* and *U. urealyticum* / *U. parvum* [39.7% (29/73)]. A study by Zhu *et al*. (2012) in female patients with genital tract infections admitted to the gynaecological department of Changzhou Fourth Hospital Soochow University and Changzhou Tumor Hospital in China, between January 2005 and December 2009 reported *M. hominis* and *U. urealyticum* / *U. parvum* mixed infections of 13.91% and *M. hominis* mono-infection of 1.71%. In a study conducted by Seifoleslami *et al*., (2013) in Iran who collected a total of 150 high vaginal swab samples from the infertile females and 200 high vaginal swab samples from fertile females, mono-infection of *M. hominis* and *U. urealyticum* was detected in 3.14% and 4.28% respectively. Seifoleslami *et al*. (2013) reported 1.42% mixed infections of *M. hominis* and *U. urealyticum*. In their study, the authors classified infertility as, a female who was not pregnant after a year of regular intercourse without prevention (Seifoleslami *et al*., 2013). Bayraktar *et al.* (2010) and Redelinghuys *et al.* (2014) reported *M. hominis* and *U. parvum* of up to 80%, which might indicate that these genital mycoplasmas are colonisers found in the vaginal milieu (Bayraktar *et al.,* 2010; Redelinghuys *et al.,* 2014).

Chlamydia trachomatis, N. gonorrhoeae and *T. vaginalis* were also investigated in this study using the Anyplex II STI-7 PCR (Seegene, Seoul, Korea) assay. In this study, an overall prevalence of 11% *T. vaginalis*, 6% *Chlamydia trachomatis* and 1% *N. gonorrhoeae* was observed. This is of a great concern as the presence of the above-mentioned pathogens in pregnancy may result in substantial foetal and neonatal morbidity and mortality (Morikawa *et al*., 2018). While some countries recommend universal diagnostic testing for STIs as a component of antenatal care, theWorld Health Organization (WHO) recommends symptomatic screening with syndromic management for STIs in resource-limited settings (Newman *et al*., 2015; Adachi, *et al*., 2016; Medline *et al*., 2017). However, given that most STIs are asymptomatic, the majority of women remain undiagnosed and untreated for their infection(s) (White *et al*., 2008). Physiological changes during pregnancy, such as changes in vaginal discharge and urinary habits, may further mask the signs and symptoms of a true infection (White *et al*., 2008). Wangnapi *et al*. (2014) reported prevalence rates of 21.3% (77/362) for *T. vaginalis*, 11% (40/362) for *C. trachomatis* and 10% (35/362) for *N. gonorrhoeae* in a cross-sectional survey of 400 pregnant women at their first visit at a rural or urban antenatal clinic (ANCs) in Madang, a province located along the North coast of Papua New Guinea, between February 2011 and April 2012. The discrepancy in the rates between our findings and that of Wangnapi *et al*. (2014) can be attributed to the fact that women in our study have access to healthcare and have been looked after by specialists who have been monitoring them, while the women participating in the Wangnapi *et al*. (2014) study were screened at their fist visit to the antenatal clinics. A study conducted by Peters *at al*. (2014) at 25 of the 100 primary healthcare (PHC) facilities across the Mopani District, South Africa in rural areas where there is not access to good healthcare observed a high prevalence of genital chlamydial infection (16%) and gonorrhoea (10%).

A prevalence of 11% for *T. vaginalis* was observed in this study. De Jongh *et al.* (2010) who conducted a study on women presenting at a termination of pregnancy clinic in Pretoria, South Africa reported a prevalence of 12.5% for *T. vaginalis.* Crucitti *et al*. (2010) conducted a study in Ndola, Zambia, which included adolescent girls attending school, pregnant women attending an antenatal clinic, and commercial sex workers (CSW) and reported prevalence rates for *T. vaginalis* in the adolescent girls, pregnant women, and among the commercial sex workers of 25%, 32% and 33.2% respectively. The other major concern is that they detected *T. vaginalis* among adolescent girls who denied that they ever had sexual intercourse; prevalence reported of 22.9% (87/380) (Crucitti *et al*., 2010). Naidoo and Wand (2013) who analysed data from women participating in a phase III vaginal diaphragm trial conducted in two communities in Durban, South Africa from 2003 to 2006 reported a lower prevalence of 6.2% for *T. vaginalis*.

Various factors contribute to the vulnerability of African women for STIs. Economic dependency on men, inability to negotiate condom use, concurrent sexual relationships, engagement in transactional sex for survival, non-specific clinical symptoms, high rate of asymptomatic infections and poor knowledge of and access to STI treatment services, contribute to the high prevalence of STIs observed in our study (Peters *et al*., 2014). Another factor that may lead to a higher number of cases might be the high migration rate of partners from rural areas to urban areas in search of employment where they have sexual relationships with other partners (Heng and Liebmann, 2000; Wang, *et al*., 2010).

A higher prevalence of all seven STIs was detected in the antenatal clinic compared to the reproductive biology clinic. None of the participants attending the reproductive biology clinic was positive for *M. genitalium, N. gonorrhoeae* or *T. vaginalis*. The use of a molecular technique (Anyplex II STI-7 real-time PCR assay) enabled us to detect seven STIs simultaneously and detect key pathogens such as *C. trachomatis*, *M. genitalium, N. gonorrhoeae* and *T. vaginalis* in both clinics. Generally, the key STI pathogens are often asymptomatic or responsible for non-specific symptoms: therefore, if undiagnosed, they will lead to reproductive sequelae or complications in the upper genital tract (Del Prete *et al*., 2017). The number of married people is higher in the reproductive biology (Obstetrics and Gynecology) clinic compared to an antenatal clinic. The high number of married people in the reproductive biology clinic might have contributed to the lower percentage of STIs in this clinic because being married reduces the risky behaviour of having multiple sexual partners.

4.6 Conclusion

This study confirms the high prevalence of *U. parvum* among women visiting an antenatal and reproductive biology clinic. Routine testing of STIs is encouraged for the identification and management of infections especially in pregnant and infertile women as this can lead to severe reproductive complications and adverse pregnancy outcomes because some of the women are assymtomatic. 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 as it can detect seven STIs simultaneously.

Acknowledgements

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Table 4.1: Summary of the demographics of the patients obtained from an antenatal and reproductive biology clinic

Variables	Clinic name	
	Antenatal	**Reproductive biology**
Age	29.36 (19-43)	32 (20-43)
Ethnicity		
African	44 (80%)	41(80%)
White	10 (18%)	6(12%)
Caucasian	1 (2%)	4(8%)
Marital status		
Married	22(40%)	43(84%)-
Single	33(60%)	8(16%)
Divorced	0	0
Gestational weeks	28.13(5-39)	-
***HIV* status**		
Positive	11(20%)	7(14%)
Negative	44(80%)	44(86%
Tampon *use*		
Yes	3(5%)	5(10%)
No	52(95%)	46(90%)
New sexual partners (last 6 months*)*		
Yes	1(2%)	1(2%)
No	54(98%)	50(98%)
Douching		
Yes	4(7%)	8(16%)-
No	51(93%)	43(84%)
Condom use		
Always	0	2(4%)
Yes	18(33%)	4(8%)
No	37(67%)	43(84%)
Alcohol consumption		
Yes	1(2%)	13(25%)
No	54(98%)	38(75%)
Vaginal sex frequency	1.4(0-5)	2.3(0-4)

Table 4.2: Prevalence of genital mycoplasmas and other co-infections using found in the two clinics using Anyplex II STI-7 PCR

Pathogens	Clinic name		
	Antenatal N=55 N (%)	Reproductive biology N=51 N (%)	Overall n=106 N (%)
Mycoplasma hominis			
Positive	20 (36%)	10 (20%)	30 (28%)
Negative	35 (64%)	41 (80%)	76 (72%)
Mycoplasma genitalium			
Positive	2 (4%)	0	2 (2%)
Negative	53 (96%)	51 (100%)	104 (98%)
Ureaplasma urealyticum			
Positive	18(33%)	8 (16%)	26 (25%)
Negative	37(67.3%)	43 (84%)	80 (76%)
Ureaplasma parvum			
Positive	40 (73%)	28 (55%)	68 (64%)
Negative	15 (27%)	23 (45%)	38 (36%)
Chlamydia trachomatis			
Positive	5 (9%)	1 (2.0%)	6 (5.7%)
Negative	50 (91%)	50 (98%)	100 (94%)
Neisseria gonorrhoeae			
Positive	1 (2%)	0	1 (1%)
Negative	54 (98%)	51 (100%)	105 (99%)
Trichomonas vaginalis			
Positive	12 (22%)	0	12 (11%)
Negative	43 (78%)	51 (100%)	94 (89%)

CHAPTER 5

Concluding remarks

5.1 Conclusions

Sexually transmitted infections (STIs) contribute to the disease burden on the health system in South Africa; these infections are recognized as a major contributor to the human immunodeficiency virus (HIV) epidemic (Naidoo *et al.*, 2014). High STI prevalence rates among young people have been observed worldwide and highlights the critical need for global efforts to improve sexual and reproductive health in this population (Francis *et al.*, 2018). Sexually transmitted infections can cause acute conditions such as cervicitis, urethritis, and genital ulceration (Naidoo *et al.*, 2014). These infections can also lead to severe complications and long term sequelae, including pelvic inflammatory disease, ectopic pregnancy, infertility, chronic pelvic pain and neurological and cardiovascular disease in adults, neonatal death, premature delivery, blindness, or severe disability in infants, and an increased risk of HIV acquisition and transmission (Muvunyia *et al.,* 2011; Newman *et al*., 2015). Major STIs include bacterial vaginosis, *Chlamydia trachomatis, Neisseria gonorrhoeae*, *Trichomonas vaginalis* and the genital mycoplasmas (Avolio *et a*l., 2016; Vică *et al*., 2016).

Genital mycoplasmas, include *Mycoplasma genitalium*, *M hominis* and the *Ureaplasma* spp. *Mycoplasma hominis* and *Ureaplasma* spp. are potentially pathogenic bacteria that frequently colonise the genitourinary system of sexually active individuals. The most prevalent species detected was *U. parvum.* Several studies reported *U. parvum* to be the most prevalent detected genital mycoplasma in reproductive age women with prevalence rates ranging from 17% to 64% (Povlsen *et al*., 2002; Kataoka *et al*., 2006; De Francesco *et al*., 2009; Del Prete *et al.*, 2017). The high prevalence of *U. parvum* infection in women visiting an antenatal and reproductive biology clinic can negatively impact on the pregnancy or *in vitro* fertilisation (IVF). The presence of genital mycoplasmas infection in women is associated with adverse outcomes including pelvic inflammatory disease, concurrent vaginitis and adverse pregnancy outcomes and it can enhance the risk of HIV (Fichorova, 2009).

Risk factors that influence the increased rate of STIs amongst populations of developing countries have been identified as a combination of behavioural and social factors, demographics and economics (Stoneburner & Low-Beer, 2004). The lack of education, alcohol and drug abuse as well as multiple sexual partners have been recognised as significant contributing factors to the increasing rate of STIs (Flint & du Plessis, 2013). Within South Africa, an additional contributing factor to the increased rates of STIs is a common phenomenon of migration by men from rural to urban areas for employment (Flint & du Plessis, 2013).

Genital mycoplasmas were cultured using the gold standard medium, the A7 agar. The positivity detected from the A7 agar was lower with 18% (7/40) in the reproductive biology clinic and 37% (11/29) in the antenatal clinic as compared to the positivity on the Urea-Arginine LYO 2 broth. Although positivity on the Urea-Arginine LYO 2 broth may not be a true reflection of positive genital mycoplasmas as other bacteria can cause the colour change of the broth. The lower positivity rate on A7 agar media with comparison to the Mycofast Revolution assay observed in this study highlights the low sensitivity of classic culture methods. The culturing of genital mycoplasmas requires skilled laboratory personnel due to the fastidious nature of genital mycoplasmas. Many genital mycoplasmas infections may be under-detected, as these infections show no apparent symptoms; however, even when these bacteria are detected it is difficult to distinguish between colonisation and infection without any pathological threshold (Taylor-Robinson, 2007; Cheong *et al.*, 2010). The A7 agar medium expired within a very short time period, and the recruitment process of participants was slow, as a result more of the A7 agar expired. This medium was expensive and as it did not yield good results, it was decided to not further culture all the specimens.

The Mycofast Revolution assay detected more genital mycoplasmas than the A7 agar medium, which reiterates the higher sensitivity of this technique. The reason Mycofast Revolution detected higher rates of genital mycoplasmas is because it is easy to perform and it takes 24 h to 48 h to results interpretation compared to culture using A7 agar, which requires expertise and takes up to five days to grow. The higher positivity rate of genital mycoplasmas obtained using the Mycofast revolution assay indicated that the Mycofast Revolution assay might be a more acceptable assay to use than conventional culture medium. This assay may be more cost-effective in the sense of screening for positive specimens and antimicrobial susceptibility testing.

Bacterial resistance to routinely antimicrobial agents is a growing and a worldwide problem (Rahman *et al.*, 2016). The Mycofast Revolution assay, which allows for the rapid detection, identification and determination of antimicrobial susceptibility profiles of genital mycoplasmas was used in our study (Rahman *et al*., 2016). Monitoring the antimicrobial susceptibility patterns of genital mycoplasmas may assist with optimising treatment guidelines and overall improve therapeutic outcome. Nonetheless, in this study the Mycofast Revolution assay had limited capability of providing antimicrobial susceptibility profiles specific for *M. hominis*. This was because in most cases this bacterium was isolated in culture; it was mixed with *Ureaplasma* spp. Another limitation of the study was that the bacterial strains were not analysed for specific mutations where antimicrobial resistance was detected. Phenotypic antimicrobial susceptibility testing using microbroth dilution was not done to determine whether accurate results were obtained with the Mycofast Revolution assay. More studies in the Pretoria region are needed to confirm the high resistance rates of genital mycoplasmas to common antimicrobial agents and to determine the specific genetic elements responsible for resistance.

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 these assays 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. Multiplex PCR allows for faster detection and a reduction in labour (Dierkes *et al*., 2009). A multiplex commercial PCR (Anyplex™ II STI-7 Detection kit) assay was used in this study. The use of this assay in this study allowed for the simultaneous detection of seven STIs (*C. trachomatis, M. genitalium, M. hominis, N. gonorrhoeae, T. vaginalis, U. parvum* and *U. urealyticum*). This test also allowed for the differentiation between *U. parvum* and *U. urealyticum*. The use of this test allowed for detection of slow-growing species, such as *M. genitalium*, which are difficult to detect and identify using culture (Waites *et al*., 2012).

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). Shortfalls of the syndromic management approach is that it lacks 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).

A higher positivity rate for genital mycoplasmas was observed in the participants attending the antenatal clinic as compared to the reproductive biology clinic. This may be due to the fact that most participants attending the reproductive biology clinic were married women as compared to the participants attending the antenatal clinic whom were mostly single women. There is a higher possibility for a single person to engage with multiple partners; a practice that will increase the chances of being infected with STIs compared to someone who is married. Naidoo and Wand (2013) also reported that unmarried non-cohabiting women were at a higher risk of having a *T. vaginalis* infection. Research suggests that HIV-positive individuals with an STI may be at an increased risk of passing HIV to someone else through anal, vaginal and frontal sex (Johnson and Lewis, 2008).

Several limitations were identified in this study: (i) the samples size was too small to make generilisable conclusions on the prevalence of STIs in the area and populations studied; (ii) no follow-up specimens were taken from the included participants, and (iii) the conventional multiplex PCR detected a low number of positive genital mycoplasmas compared to Mycofast Revolution assay, which indicates that there was an operational error by the principal investigator, while perfoming the technique.

Following the findings of the current study using the Mycofast revolution and Anyplex II STI-7 PCR (Seegene, Seoul, Korea), it is recommended that point of care testing for the rapid identification and treatment of genital mycoplasmas in pregnant women should be considered by local healthcare providers. Patients should be screened at the first antenatal clinic visit for genital mycoplasmas and other STIs. This screening may identify asymptomatic cases and correctly identify symptomatic cases. This can then be followed by treatment of the patients with the appropriate antimicrobial agents.

5.2 Future research

The findings of this study supported 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. Sensitive molecular assays, such as real time PCR assays and the Mycofast

Revolution assay are important assays for research purposes and diagnostics. The molecular assays can be used for diagnosis but the main problem might be the cost as the reagents to perfom the tests are expensive. However, point of care (POC) testing, which is defined as near-patient testing in a hospital, doctor's office, clinic, or home, offers advantages when a rapid answer is required (Niemz *et al*., 2011). 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 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 sequencing of genomes and mechanisms of antimicrobial resistance of genital mycoplasmas. Genetic analyses of the genes conferring antimicrobial resistance may give a better indication of which genes or mutations are responsible for resistance as genital mycoplasmas are prone to have point mutations (Krausse and Schubert, 2010; Chrisment *et al*., 2012).

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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, US)	93.05 g
Ultrapure water	400 mL
Sodium hydroxide (NaOH) pellets (MerckMillipore, US)	

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, US)	27 g
Boric acid (Sigma-Aldrich, US)	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. 50% Glycerol solution for long-term storage

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

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

4. 2% (m/v) Agarose gels

MetaPhor™ agarose powder (Lonza, US)	2 g

1X TBE buffer	100 mL
Ethidium bromide [10 mg/mL (Sigma-Aldrich, US)]	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, insert 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 Bashing BeadTM (Zymo Research, US) Lysis tube.

2. Add 750 µL of lysis solution and vortex (Disruptor Genie, Scientific Inductries, Inc., US) at maximum speed for 5 min

3. Centrifuge at 10 000 *g* (Spectrafuge™ 24D, Labnet International Inc, US) for 1 min

4. Add 400 µL of supernatant to Zymo-SpinTM IV Spin Filter (orange top) (Zymo Research, US) 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, US) for 1 min

5. Add 1 200 µL of Fungal/Bacterial DNA binding buffer [with 0.5% 2-mercaptoethanol Merck Millipore, US)] to the supernatant

6. Transfer 800 µL of the supernatant to Zymo-SpinTM IIC Spin Column (Zymo Research, US) in a collection tube and centrifuge at 10 000 *g* (Spectrafuge™ 24D, Labnet International Inc, US) for 1 min. Discard the flow. Repeat step again

7. Add 200 µL of DNA Pre-wash Buffer to Zymo-SpinTM IIC Spin column (Zymo Research, US) in a new collection tube and centrifuge at 10 000 *g* (Spectrafuge™ 24D, Labnet International Inc, US) for 1 min

8. Add 500 µL of Fungal/Bacterial DNA wash buffer to Zymo-SpinTM IIC Spin column (Zymo Research, US) and centrifuge at 10 000 *g* for 1 min

9. Transfer the Zymo-SpinTM IIC Spin column (Zymo Research, US) to a clean 2 mL microcentrifuge tube (Axygen, Corning, US) and add 100 µL of DNA elution buffer

10. Centrifuge at 10 000 *g* (Spectrafuge™ 24D, Labnet International Inc, US) for 30 sec to elute DNA

APPENDIX C

RESULTS FOR DIAGNOSTICS TESTS PERFORMED AND CLINICAL DATA OF PATIENTS

Table 1: Results for diagnostics tests performed at an antenatal clinic

Anyplex Real time PCR results								Mycofast Revolution		Mycoplasma Culture	Mycoplasma Culture	Bacterial vaginosis
Code	**UP**	**UU**	**MG**	**MH**	**CT**	**TV**	**NG**	**Ureplasma spp (UU/UP)**	**M.hominis (MH)**	**unine broth**	**A7 AGAR**	**Comments**
P1	POS	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	POS	NEG	Negative BV
P2	NEG	POS	NEG	NEG	NEG	NEG	NEG	POS	NEG	POS	NEG	Negative BV
P3	POS	POS	POS	NEG	NEG	NEG	NEG	POS	NEG	POS	NEG	Negative BV
P4	POS	POS	NEG	NEG	NEG	NEG	NEG	POS	NEG	NEG	-	Intermediate
P5	POS	NEG	POS	NEG	NEG	NEG	NEG	POS	NEG	POS	NEG	Negative BV
P6	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	Negative BV
P7	POS	NEG	NEG	NEG	NEG	POS	NEG	POS	NEG	POS	NEG	Negative BV
P8	POS	NEG	NEG	POS	NEG	NEG	NEG	POS	POS	POS	NEG	Positive BV
P9	POS	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	POS	NEG	Negative BV
P10	NEG	POS	NEG	NEG	NEG	NEG	NEG	POS	NEG	POS	POS	Negative BV
P11	POS	NEG	NEG	POS	NEG	NEG	NEG	POS	POS	POS	POS	Negative BV
P12	POS	NEG	NEG	POS	NEG	NEG	NEG	POS	POS	POS	POS	Positive BV
P13	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	Intermediate
P14	POS	POS	NEG	POS	NEG	NEG	NEG	POS	POS	POS	POS	Positive BV
P15	POS	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	POS	NEG	Negative BV
P16	POS	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	POS	NEG	Negative BV
P17	POS	NEG	NEG	POS	NEG	NEG	NEG	POS	NEG	POS	NEG	Intermediate
P18	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	Positive BV
P19	POS	NEG	NEG	POS	NEG	NEG	NEG	POS	POS	POS	POS	Negative BV
P20	NEG	POS	NEG	POS	POS	NEG	POS	POS	POS	POS	POS	Positive BV
P21	NEG	POS	NEG	POS	NEG	NEG	NEG	POS	POS	POS	POS	Negative BV
P22	POS	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	POS	NEG	Negative BV
P23	NEG	POS	NEG	NEG	NEG	NEG	NEG	POS	NEG	POS	POS	Negative BV
P24	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	Negative BV
P25	POS	POS	NEG	NEG	NEG	POS	NEG	POS	NEG	POS	NEG	Negative BV
P26	NEG	NEG	NEG	POS	NEG	NEG	NEG	POS	POS	POS	POS	Negative BV
P27	POS	NEG	NEG	POS	NEG	NEG	NEG	POS	POS	POS	POS	Positive BV
P28	POS	NEG	NEG	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	Intermediate
P29	POS	POS	NEG	POS	POS	NEG	NEG	POS	NEG	POS	NEG	Intermediate
P30	POS	POS	NEG	NEG	NEG	POS	NEG	POS	NEG	POS	POS	Intermediate
P31	POS	POS	NEG	NEG	NEG	NEG	NEG	POS	NEG	POS	N/A	Negative BV
P32	POS	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	POS	N/A	Negative BV
P33	POS	NEG	NEG	POS	POS	NEG	NEG	POS	POS	POS	N/A	Negative BV
P34	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	N/A	Negative BV
P35	POS	NEG	NEG	NEG	POS	POS	NEG	POS	NEG	POS	N/A	Positive BV

NEG-NEGATIVE, POS-POSITIVE; MG- *Mycoplasma hominis, UU- Ureaplasma urealyticum, UU- Ureaplasma parvum*, MG- *Mycoplasma genitalium*, CT- *Chlamydia trachomatis*, NG- *Neisseria gonorrhoeae*, TV- *Trichomonas vaginalis*

Table 1: Results for diagnostics tests performed at an antenatal clinic (continued)

Anyplex Real time PCR results								Mycofast Revolution		Mycoplasma Culture	Mycoplasma Culture	Bacterial vaginosis
Code	**UP**	**UU**	**MG**	**MH**	**CT**	**TV**	**NG**	**Ureplasma spp (UU/UP)**	**M.hominis (MH)**	**unine broth**	**A7 AGAR**	**Comments**
P36	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	N/A	Intermediate
P37	POS	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	POS	N/A	Intermediate
P38	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	N/A	Positive BV
P39	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	N/A	Negative BV
P40	NEG	POS	NEG	POS	NEG	NEG	NEG	POS	POS	POS	N/A	Negative BV
P41	POS	NEG	NEG	POS	NEG	POS	NEG	NEG	POS	POS	N/A	Negative BV
P42	POS	POS	NEG	NEG	NEG	POS	NEG	NEG	NEG	POS	N/A	Intermediate
P43	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	N/A	Negative BV
P44	POS	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	N/A	Negative BV
P45	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	N/A	Intermediate
P46	POS	NEG	NEG	NEG	NEG	POS	NEG	NEG	NEG	NEG	N/A	Positive BV
P47	POS	NEG	NEG	NEG	NEG	POS	NEG	NEG	NEG	POS	N/A	Negative BV
P48	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	N/A	Intermediate
P49	NEG	POS	NEG	NEG	NEG	NEG	NEG	POS	NEG	POS	N/A	Intermediate
P50	POS	POS	NEG	POS	POS	NEG	NEG	NEG	POS	POS	N/A	Positive BV
P51	POS	NEG	NEG	POS	NEG	POS	NEG	NEG	NEG	POS	N/A	Negative BV
P52	POS	NEG	NEG	NEG	NEG	POS	NEG	POS	NEG	POS	N/A	Negative BV
P53	POS	NEG	NEG	POS	NEG	NEG	NEG	POS	POS	POS	N/A	Intermediate
P54	POS	POS	NEG	POS	NEG	POS	NEG	POS	POS	POS	N/A	Negative BV
P55	POS	NEG	NEG	POS	NEG	POS	NEG	NEG	NEG	POS	N/A	Positive BV

NEG-NEGATIVE, POS-POSITIVE; MG- *Mycoplasma hominis, UU- Ureaplasma urealyticum, UU- Ureaplasma parvum*, MG- *Mycoplasma genitalium*, CT- *Chlamydia trachomatis*, NG- *Neisseria gonorrhoeae*, TV- *Trichomonas vaginalis*

Table 2: Results for diagnostics tests performed at reproductive biology clinic

Anyplex Real time PCR results								Mycofast Revolution		Mycoplasma Culture	Mycoplasma culture	Bacterial vaginosi
Code	**UP**	**UU**	**MG**	**MH**	**CT**	**TV**	**NG**	**Ureplasma spp(UU/UP)**	***M.hominis* (MH)**	**unine broth**	**A7 AGAR**	**Comments**
I1	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	Negative BV
I2	POS	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	POS	NEG	Negative BV
I3	POS	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	POS	NEG	Intermediate
I4	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	Negative BV
I5	POS	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	POS	NEG	Negative BV
I6	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	Negative BV
I7	POS	NEG	NEG	POS	NEG	NEG	NEG	POS	POS	POS	NEG	Positive BV
I8	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	Negative BV
I9	POS	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	POS	POS	Negative BV
I10	POS	NEG	NEG	NEG	POS	NEG	NEG	POS	NEG	POS	POS	Positive BV
I11	POS	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	POS	NEG	Negative BV
I12	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	Negative BV
I13	POS	NEG	NEG	POS	NEG	NEG	NEG	POS	POS	POS	POS	Positive BV
I14	NEG	NEG	NEG	POS	NEG	NEG	NEG	NEG	POS	NEG	NEG	Negative BV
I15	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	Negative BV
I16	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	Intermediate
I17	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	Negative BV
I18	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	Positive BV
I19	POS	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	POS	NEG	Intermediate
I20	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	Intermediate
I21	POS	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	POS	NEG	Negative BV
I22	NEG	NEG	NEG	POS	NEG	NEG	NEG	NEG	POS	POS	POS	Positive BV
I23	NEG	POS	NEG	POS	NEG	NEG	NEG	POS	POS	POS	POS	Negative BV
I24	POS	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	POS	NEG	Positive BV
I25	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	Negative BV
I26	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	Intermediate
I27	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	Negative BV
I28	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	Negative BV
I29	POS	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	POS	NEG	Negative BV
I30	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	Intermediate
I31	POS	NEG	NEG	POS	NEG	NEG	NEG	POS	POS	POS	NEG	Negative BV
I32	POS	NEG	NEG	POS	NEG	NEG	NEG	POS	POS	POS	NEG	Negative BV
I33	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	Positive BV
I34	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	Intermediate
I35	POS	POS	NEG	NEG	NEG	NEG	NEG	POS	NEG	POS	NEG	Intermediate
I36	POS	POS	NEG	NEG	NEG	NEG	NEG	POS	NEG	POS	NEG	Negative BV
I37	POS	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	POS	NEG	Negative BV
I38	NEG	POS	NEG	NEG	NEG	NEG	NEG	POS	NEG	NEG	NEG	Negative BV
I39	POS	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	POS	NEG	Negative BV
I40	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	Negative BV
I41	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	N/A	Positive BV
I42	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	N/A	Intermediate
I43	POS	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	POS	N/A	Intermediate
I44	POS	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	POS	N/A	Negative BV
I45	NEG	POS	NEG	POS	NEG	NEG	NEG	POS	POS	POS	N/A	Positive BV
I46	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	N/A	Negative BV
I47	POS	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	POS	N/A	Positive BV
I48	NEG	POS	NEG	POS	NEG	NEG	NEG	POS	POS	POS	N/A	Negative BV
I49	POS	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	POS	N/A	Intermediate
I50	NEG	POS	NEG	POS	NEG	NEG	NEG	POS	NEG	POS	N/A	Negative BV
I51	NEG	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	N/A	Negative BV

NEG-NEGATIVE, POS-POSITIVE; MG- *Mycoplasma hominis, UU- Ureaplasma urealyticum, UU- Ureaplasma parvum*, MG- *Mycoplasma genitalium*, CT- *Chlamydia trachomatis*, NG- *Neisseria gonorrhoeae*, TV- *Trichomonas vaginalis*

Table 3: Results for Mycofast revolution performed at an antenatal clinic

Sample	LVX			MXF		E		CM		TE					
	1	2	4	0.25	2	8	16	0.3	0.5	1	2	4	8	Ureplasma spp(UU/UP)	M.hominis(MH)
P1	NEG	NEG	POS	POS	NEG	POS	NEG	POS	POS	POS	POS	POS	POS	POS	NEG
P2	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	NEG	NEG	NEG	NEG	POS	NEG
P3	POS	NEG	NEG	POS	NEG	POS	NEG	POS	POS	POS	POS	POS	POS	POS	NEG
P4	POS	NEG	NEG	POS	POS	NEG	NEG	POS	POS	NEG	NEG	NEG	POS	POS	NEG
P5	NEG	NEG	NEG	POS	NEG	POS	NEG	POS	POS	POS	POS	POS	POS	POS	NEG
P6	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
P7	POS	POS	NEG	POS	NEG	POS	NEG	POS	POS	POS	POS	NEG	NEG	POS	NEG
P8	POS	NEG	NEG	NEG	NEG	NEG	NEG	POS	POS	POS	POS	POS	NEG	POS	POS
P9	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	NEG	NEG	POS	NEG	POS	NEG
P10	POS	NEG	NEG	POS	NEG	POS	NEG	POS	POS	NEG	NEG	NEG	NEG	POS	NEG
P11	POS	POS	POS	NEG	NEG	NEG	NEG	POS	POS	POS	POS	POS	POS	POS	POS
P12	POS	NEG	NEG	POS	NEG	POS	NEG	POS	POS	POS	NEG	NEG	NEG	POS	POS
P13	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
P14	POS	POS	NEG	NEG	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS
P15	POS	NEG	NEG	POS	NEG	POS	NEG	POS	POS	NEG	NEG	NEG	NEG	POS	NEG
P16	POS	POS	POS	POS	NEG	NEG	NEG	POS	POS	NEG	NEG	NEG	NEG	POS	NEG
P17	POS	NEG	NEG	POS	NEG	NEG	NEG	POS	POS	NEG	NEG	NEG	NEG	POS	NEG
P18	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
P19	POS	NEG	NEG	POS	NEG	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS
P20	POS	POS	NEG	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS
P21	POS	POS	NEG	POS	NEG	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS
P22	POS	NEG	NEG	POS	POS	POS	NEG	POS	POS	NEG	NEG	NEG	NEG	POS	NEG
P23	POS	NEG	NEG	POS	POS	NEG	NEG	POS	NEG	NEG	NEG	NEG	NEG	POS	NEG
P24	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
P25	POS	POS	NEG	POS	NEG	POS	NEG	POS	POS	POS	POS	POS	POS	POS	NEG
P26	POS	POS	POS	POS	NEG	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS
P27	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	POS	NEG	NEG	NEG	NEG	POS	POS
P28	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
P29	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	NEG	NEG	NEG	NEG	POS	NEG
P30	POS	NEG	NEG	POS	NEG	POS	NEG	POS	POS	POS	POS	POS	POS	POS	NEG
P31	NEG	NEG	NEG	POS	POS	POS	POS	POS	POS	POS	POS	POS	NEG	POS	NEG
P32	POS	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	NEG	NEG	NEG	NEG	POS	NEG

NEG-NEGATIVE, POS-POSITIVE, LVX - Levofloxacin , MXF - Moxifloxacin , E- Erythromycin, CM- Clindamycin, TE- Tetracycline

Table 3: Results for Mycofast revolution performed at an antenatal clinic (continued)

Sample	LVX			MXF		E		CM		TE					
	1	2	4	0.25	2	8	16	0.3	0.5	1	2	4	8	Ureplasma spp(UU/UP)	M.hominis(MH)
P33	POS	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	NEG	NEG	NEG	NEG	POS	POS
P34	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
P35	POS	POS	NEG	NEG	NEG	NEG	NEG	POS	POS	POS	POS	POS	NEG	POS	NEG
P36	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
P37	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	POS	NEG	NEG	NEG	NEG	POS	NEG
P38	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
P39	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
P40	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	POS	NEG	NEG	NEG	NEG	POS	POS
P41	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS
P42	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
P43	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
P44	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
P45	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
P46	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
P47	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
P48	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
P49	NEG	NEG	NEG	NEG	NEG	NEG	POS	POS	POS	NEG	NEG	NEG	NEG	POS	NEG
P50	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	POS	NEG	NEG	NEG	POS	NEG	POS
P51	NEG	NEG	NEG	POS	POS	POS	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
P52	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	POS	POS	POS	NEG	NEG	POS	NEG
P53	POS	POS	NEG	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS
P54	POS	POS	NEG	NEG	NEG	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS
P55	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG

NEG-NEGATIVE, POS-POSITIVE; LVX - Levofloxacin , MXF - Moxifloxacin , E- Erythromycin, CM- Clindamycin, TE- Tetracycline

Table 4: Results for Mycofast revolution performed at the reproductive biology clinic

Sample	LVX			MXF		E		CM		TE				Mycofast Revolution	
	1	2	4	0.25	2	8	16	0.3	0.5	1	2	4	8	Ureplasma spp(UU/UP)	M.hominis(MH)
I1	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
I2	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	POS	POS	POS	POS	POS	NEG
I3	POS	POS	POS	POS	NEG	NEG	NEG	POS	POS	NEG	NEG	NEG	NEG	POS	NEG
I4	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
I5	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	POS	POS	POS	POS	POS	POS	NEG
I6	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
I7	POS	NEG	POS	POS	NEG	POS	POS	POS	POS	NEG	NEG	NEG	NEG	POS	POS
I8	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
I9	NEG	NEG	NEG	POS	NEG	POS	POS	POS	POS	POS	POS	POS	POS	POS	NEG
I10	NEG	NEG	NEG	POS	NEG	NEG	NEG	POS	POS	NEG	NEG	NEG	NEG	POS	NEG
I11	NEG	NEG	NEG	POS	NEG	NEG	NEG	POS	POS	NEG	POS	NEG	NEG	POS	NEG
I12	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
I13	NEG	NEG	NEG	POS	NEG	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS
I14	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	NEG	NEG	NEG	NEG	NEG	POS
I15	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
I16	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
I17	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
I18	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
I19	POS	NEG	NEG	POS	NEG	POS	NEG	POS	POS	NEG	NEG	NEG	NEG	POS	NEG
I20	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
I21	POS	NEG	NEG	POS	NEG	NEG	NEG	POS	POS	NEG	NEG	NEG	NEG	POS	NEG
I22	NEG	NEG	NEG	NEG	NEG	POS	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS
I23	NEG	NEG	NEG	POS	NEG	NEG	NEG	POS	POS	POS	POS	POS	POS	POS	POS
I24	POS	NEG	NEG	POS	POS	NEG	NEG	POS	POS	POS	POS	POS	POS	POS	NEG
I25	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
I26	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
I27	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
I28	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
I29	POS	NEG	NEG	POS	NEG	POS	NEG	POS	NEG	POS	NEG	NEG	NEG	POS	NEG
I30	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
I31	POS	NEG	NEG	POS	NEG	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS
I32	POS	NEG	NEG	POS	NEG	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS
I33	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
I34	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
I35	POS	POS	NEG	POS	NEG	POS	POS	POS	POS	POS	POS	POS	NEG	POS	NEG
I36	POS	POS	NEG	POS	NEG	POS	POS	POS	POS	POS	POS	POS	POS	POS	NEG
I37	POS	NEG	NEG	POS	NEG	POS	POS	POS	POS	POS	NEG	NEG	NEG	POS	NEG
I38	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	NEG	NEG	NEG	NEG	POS	NEG
I39	POS	NEG	NEG	POS	NEG	POS	POS	POS	POS	POS	NEG	NEG	NEG	POS	NEG
I40	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
I41	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
I42	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
I43	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	POS	POS	POS	NEG	POS	NEG
I44	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG
I45	NEG	POS	NEG	POS	NEG	NEG	NEG	POS	POS	POS	POS	POS	NEG	POS	POS
I46	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
I47	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG	POS	NEG	POS	NEG	POS	NEG
I48	NEG	POS	NEG	NEG	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS
I49	NEG	NEG	NEG	NEG	NEG	NEG	POS	POS	POS	NEG	NEG	NEG	NEG	POS	NEG
I50	NEG	NEG	NEG	POS	NEG	POS	NEG	POS	POS	POS	NEG	NEG	NEG	POS	NEG
I51	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG

NEG-NEGATIVE, POS-POSITIVE; LVX - Levofloxacin , MXF - Moxifloxacin , E- Erythromycin, CM- Clindamycin, TE- Tetracycline

Table 5: Patient's demographics obtained from participants at an antenatal clinic

Date	Lab code	Patient number	Age	Ethnicity	Marital status	Parity (Births)	Gravidity (prenancys)	Pregnancy losses	Gestational weeks	RPR status	HIV Status	Tampon use	New partner	Vaginal sex frequency	Last intercourse	Douching	Frequency	BV discharge	Condom use	Medication	History of pregnancy implications	Alcohol consumption
04/06/2015	P1	K044	35	African	Single	1	3	2	12	Neg	Pos	No	No	1	7	No	NA	Present (unknown)	yes	Kidney medication	NA	No
11/06/2015	P2	GP63217071	21	White	Engaged	0	2	1	34	Neg	Neg	Yes	No	3	3	No	NA	None	No	Rennies	NA	No
04/06/2015	P3	GT63843570	28	White	Single	2	4	1	35	Neg	Neg	No	No	5	1	No	NA	None	No	None	NA	No
29/06/2016	P4	GP63173094	39	African	Married	0	2	1	38	Neg	Neg	No	No	2	4	No	NA	white discharge	No			No
11/06/2015	P6	GP63066786	29	African	Married	3	4	0	20	Neg	Neg	No	No	1	7	No	NA	Present (white, no smell)	No	Thyroid tablet	NA	No
30/06/2015	P5	GT63987132	23	African	Single	0	3	2	15	Neg	Neg	Yes	No	3	8	No	NA	(yellowish, no smell)	No	Asprin	2 miscariiges at 4 months Hanuary 2015, 10 weeks 2013	No
30/06/2015	P6	GP45890551	37	African	Married	2	3	0	31	Neg	Neg	No	No	2	3	No	NA	None	No	Vitamins	NA	No
11/06/2015	P7	GT45896961	40	African	Single	0	1	0	34	Neg	Neg	No	No	2	3	No	NA	None	No	None	NA	No
11/06/2015	P8	GT63979028	26	African	Single	3	4	0	28	Neg	Neg	No	No	1	3	No	NA	Present (fish odour)	yes	Calcium, folic acid	NA	No
11/06/2015	P9	GP63218812	41	White	Married	5	8	2	18	Neg	Neg	No	No	2	5	No	NA	None	No	Altroxin, Vitamins	NA	No
11/06/2015	P10	GT42583529	24	African	Single	0	1	0	29	Neg	Neg	No	No	1	32	Yes	Everyday	None	yes	Vitamins	NA	No
18/06/2016	P11	GP63217330	22	white	Single	0	2	2	21	Neg	Neg	No	No	2	4	No	NA	None	No			No

Table 5: Patient’s demographics obtained from participants at an antenatal clinic (continued)

Date	Lab code	Patient number	Age	Ethnicity	Marital status	Parity (Births)	Gravidity (prenancys)	Pregnancy	Gestational weeks	RPR status	HIV Status	Tampon use	New partner	Vaginal sex frequency	Last intercourse	Douching	Frequency	BV discharge	Condom use	Medication	History of pregnancy implications	Alcohol consumption
30/06/2015	P12	GP63220012	32	African	Married	0	5	4	26	Neg	Pos	No	No	1	3	Yes	Everyday	Brown, no smell	yes	ARV`s	4 pregnancy loses	No
29/06/2016	P13	GP63242378	34	African	Engaged	0	1	0	34	Neg	Neg	No	No	2	4	No	NA	yellow discharge,itchy and smelly	No			No
01/07/2015	P14	GP63213318	23	African	Single	1	2	0	31	Neg	Neg	No	No	1	33	No	NA	(no smeel, white, no itch)	yes	Vitamins	NA	yes
06/07/2015	P15	GP63217146	19	white	Single	1	2	0	21	Neg	Neg	No	No	1	14	No	NA	white, no itch, no smell	No	Vitamins	NA	No
06/07/2015	P16	Gp42449744	42	White	Married	9	11	1	24	Neg	Neg	No	No	1	35	No	NA	None	No	Vitamins	NA	No
06/07/2015	P17	GP63220400	27	White	Married	1	2	0	29	Neg	Neg	No	No	1	2	No	NA	None	No	Vitamins	NA	No
06/07/2015	P18	GT63994117	25	African	Married	1	2	0	30	Neg	Neg	No	No	1	-	No	NA	White, no smell	No	None	NA	No
30/07/2015	P19	GP63221295	32	African	Married	2	3	1	31	Neg	Pos	No	No	2	0	No	NA	light yellow, urine smell	yes	ARVS, vitamens	Termination of pregnancy	No
30/07/2015	P20	GP63191176	32	African	Single	2	4	2	34	Neg	Pos	No	yes	2	1	No	NA	None	yes	ARVS	2 miscarriges, 2 died after birth	No

Table 5: Patient's demographics obtained from participants at an antenatal clinic (continued)

Date	Lab code	Patient number	Age	Ethnicity	Marital status	Parity (Births)	Gravidity (prenancys)	Pregnancy losses	Gestational weeks	RPR status	HIV Status	Tampon use	New partner	Vaginal sex frequency	Last intercourse	Douching	Frequency	BV discharge	Condom use	Medication	History of pregnancy implications	Alcohol consumption
30/07/2015	P21	GT63994958	37	African	Single	0	4	3	28	Neg	Pos	No	No	2	5	No	NA	None	yes	arvs	1 ectopic, 2 premature babies	No
30/07/2015	P22	GP63122616	29	African	Married	0	3	2	28	Neg	Pos	No	No	3	2	No	NA	None	yes	None	1 miscarrige, 1 still birth	No
30/07/2015	P23	GT63854423	29	African	Married	1	5	3	29	Neg	Neg	No	No	2	7	No	NA	white, no itchy, smell	yes	ARVs	3 miscarriges	No
27/08/2015	P24	GP63241197	34	African	Married	2	3	0	33	Neg	Neg	No	No	1		No	NA		No			No
27/08/2015	P25	GP63216939	22	black	Single	0	0	0	32	Neg	Pos	Both	No	0	2	No	NA	creamy, no smell	No	ARVS		No
27/08/2015	P26	GP63050637	27	black	Married	1	4	2	5	Neg	Neg	No	No	3	1	No	NA	jelly like, no smell	No	Vitamins	2stillborn	No
27/08/2015	P27	GP63221197	23	black	Single	1	2	1	30	Neg	Neg	No	No	2	4	No	NA	wtery, itchy	yes	Vitamins	1 miscarrige	No
27/08/2015	P28	GT45969114	23	black	Single	2	3	0	15	Neg	-	No	No	2	14	No	NA	None	No	Vitamins	2 miscarriges	No
15/09/2015	P29	GP63221478	28	African	single	2	1	1	30	Neg	Neg	No	No	2	5	No	NA	NO	yes		N/A	No
15/09/2015	P30	GT63978762	22	coloured	Engage d	0	2	1	36	Neg	Neg	No	No	2	4	No	NA	WHITE DISCHARGE	No		N/A	No
29/04/2016	P31	GP63140035	23	white	single	2	2	0	32	Neg	Neg	No	No	4	2	No	NA	NO	No		N/A	No
29/04/2016	P32	G963235772	34	African	single	0	2	1	29	Neg	Neg	No	No	0		No	NA		No		N/A	No

Table 5: Patient's demographics obtained from participants at an antenatal clinic (continued)

Date	Lab code	Patient number	Age	Ethnicity	Marital status	Parity (Births)	Gravidity (prenancys)	Pregnancy losses	Gestational weeks	RPR status	HIV Status	Tampon use	New partner	Vaginal sex frequency	Last intercourse	Douching	Frequency	BV discharge	Condom use	Medication	History of pregnancy implications	Alcohol consumption
29/04/2016	P33	GT63881220	34	African	single	2	3	1	21	Neg	Neg	No	No	0		No	NA	yellow discharge,itchy and smelly,painful	No		N/A	No
29/04/2016	P34	GP63149231	36	African	single	3	6	2	30	Neg	Neg	No	No	0		No	NA	WHITE DISCHARGE,NOT ITCHY NO SMELL	No		N/A	No
29/04/2016	P35	GP63238520	21	African	single	0	1	0	37	Neg	Neg	No	No	0	2	No	NA	WHITE DISCHARGE	No		N/A	No
19/05/2016	P36	GP63207328	36	white	Married	0	2	1	26	Neg	Neg	No	No	2		yes	NA	none	No		N/A	No
19/05/2016	P37	GP63232935	29	African	single	0	2	1	32	Neg	Neg	No	No	1		No	NA	YEllow discharge,itchy and smelly,painful	No		N/A	No
19/05/2016	P38	GP63240683	29	African	single	2	3	2	35	Neg	Pos	No	No	3		No	NA	white discharge	yes		N/A	No
19/05/2016	P39	GT42517992	34	African	Married	1	5	3	36	Neg	Pos	No	No	1		No	NA	White discharge	yes		N/A	No
12/05/2016	P40	GP63238910	27	African	single	0	2	2	11	Neg	Neg	No	No	0		No	NA		No		N/A	No
12/05/2016	P41	GT64023767	24	African	Married	0	2	1	24	Neg	Neg	No	No	0		No	NA	Discharge. White	No		N/A	No

Table 5: Patient's demographics obtained from participants at an antenatal clinic (continued)

Date	Lab code	Patient number	Age	Ethnicity	Marital status	Parity (Births)	Gravidity (prenancys)	Pregnancy losses	Gestational weeks	RPR status	HIV Status	Tampon use	New partner	Vaginal sex frequency	Last intercourse	Douching	Frequency	BV discharge	Condom use	Medication	History of pregnancy implications	Alcohol consumption
06/06/2016	P42	GP63240403	31	African	Married	1	2	0	31	Neg	Neg	No	No	1		Yes	NA	none	yes		N/A	No
06/06/2016	P43	GT64029038	32	African	Married	0	7	0	32	Neg	Neg	No	No	2		No	NA	none	No		N/A	No
06/06/2016	P44	GP63174950	22	African	single	0	2	0	33	Neg	Neg	No	No	3		No	NA	none	No		N/A	No
09/06/2016	P45	GP63156686	25	white	single	0	4	3	36	Neg	Neg	No	No	1		No	NA	none	No		N/A	No
09/06/2016	P46	GP63226476	33	African	Married	3	7	3	34	Neg	Neg	No	No	0		No	NA	none	No		N/A	No
09/06/2016	P47	GP64022579	26	African	single	1	0	0	26	Neg	Neg	No	No	0		No	NA	DISCHARG E ,WHITE ITCHY	No		N/A	No
09/06/2016	P48	GP63164395	35	African	Married	1	2	0	33	Neg	Neg	No	No	0		No	NA	DISCHARG E ,WHITE ITCHY	No		N/A	No
09/06/2016	P49	GT63999127	25	African	single	0	2	2	23	Neg	Neg	No	No	0		No	NA	DISCHARG E ,WHITE	No		N/A	No
23/06/2016	P50	GT64028539	22	African	Married	0	2	1	37	Neg	Neg	No	No	0		No	NA	NORMAL DISCHARG E	No		N/A	No
29/06/2016	P51	GP63050878	31	African	Single	1	2	0	36	Neg	Pos	No	No	0		No	NO	Yellowish discharge	yes		N/A	No
29/06/2016	P52	GP63118425	32	African	Single	1	3	1	39	Neg	Neg	No	No	0		No	NO	none	No		N/A	No
29/06/2016	P53	GT64030064	28	African	Married	1	2	0	11	Neg	Neg	No	No	1		No	NO	none	No		N/A	No
29/06/2016	P54	GP63237220	43	African	Married	2	4	1	32	Neg	Pos	No	No	2		No	NO	DISCHARG E WHITEISH ITCHY	yes		N/A	No
29/06/2016	P55	GP63243652	27	African	Single	0	1	0	10	Neg	Neg	No	No	0		No		DISCHARG E ,WHITE	yes		N/A	No

Table 6: Patient's demographics obtained from participants at the reproductive biology clinic

Date	Sample ID	Age	Ethnicity	Marital status	Parity (Births)	Gravidity	Pregnancy losses	RPR status	HIV Status	Tampon use	New partnerlast 6 months	Vaginal sex frequency	Last intercourse(days)	Last menstruation(days)	Douching	Condom use	History of pregnancy implications	Alcohol consumption
19/05/2015	I1	29	African	Traditional	1	1	0	Neg	Neg	Yes	No	1	2	18	Yes	Always	None	NO
26/05/2015	I2	40	African	Unmarried	1	2	1	-	Neg	No	No	3	3	8	Yes	No	One pregnancy loss	NO
19/05/2015	I3	38	African	Married	0	2	2	Neg	Pos	No	No	2	4	8	No	No	2 ectopic pregnancies	NO
26/05/2015	I4	33	African	Married	0	0	0	-	Pos	No	No	3	3	23	No	Yes	NA	NO
27/05/2015	I5	37	African	Relationship	3	3	0	-	Pos	NA	No	3	61	12	Yes	Yes	3rd baby passed away	NO
09/06/2015	I6	20	Asian	Traditional	1	4	3	Neg	Neg	No	No	2	3	37	No	No	Misscarrige	NO
09/06/2015	I7	31	African	Traditional	1	3	2	Neg	Neg	No	No	3	1	35	Yes	No	NA	NO
10/06/2015	I8	30	White	Married	0	0	0	Neg	Neg	No	No	2	1	14	No	No	NA	NO
23/06/2015	I9	30	African	Married	0	3	3	-	Neg	No	No	0	-	22	No	-	Uncomforable answering Q`s	NO
23/06/2015	I10	29	African	Single	0	1	1	-	Pos	No	No	2	14	7	No	Always	NA	NO
23/06/2015	I11	36	African	Married	0	0	0	Neg	Neg	No	No	3	10	21	No	No	None	NO
01/07/2015	I12	26	White	MArried	0	0	0	Neg	Neg	No	N	1	30	21	No	No	NA	NO
01/07/2015	I13	34	African	Single	0	1	1	Neg	Neg	No	No	2	4	15	Yes	No	Miscarrige around 2007	NO
23/06/2015	I14	30	African	Married	0	3	3	-	Neg	No	No	0	-	22	No	-	Uncomforable answering Q`s	NO
06/07/2015	I15	33	White	Married	0	0	0	Neg	Neg	Yes	No	2	5	24	No	No	None	NO
21/07/2015	I16	33	African	Married	1	1	0	Neg	Neg	Yes	No	2	7	60	Yes	No	None	NO
29/07/2015	I17	37	White	Married	1	8	7	-	Neg	No	No	3	3	7	No	No	Micro-abortions	NO
28/07/2015	I18	37	African	Married	0	1	1	Neg	Pos	No	No	3	9	1	No	No	one miscarrige	YES
04/08/2015	I19	36	African	Married	0	1	1	Neg	Neg	Yes	No	1	4	14	No	No	NA	NO
04/08/2015	I20	37	African	Married	1	2	1	Neg	Neg	Yes	No	4	3	23	No	No	one miscarrige	YES
18/08/2015	I21	28	Black	Married	1	3	2	-	Neg	No	Yes	2	7	30	No	No	2 miscarriges	

Table 6: Patient's demographics obtained from participants at the reproductive biology clinic (continued)

Date	Sample ID	Age	Ethnicity	Marital status	Parity (Births)	Gravidity	Pregnancy losses	RPR status	HIV Status	Tampon use	New partnerlast 6 months	Vaginal sex frequency	Last intercourse(days)	Last menstruation(days)	Douching	Condom use	History of pregnancy implications	Alcohol consumption
18/08/2015	I22	31	Black	Married	0	0	0	-	Neg	No	no	4	14	14	No	No	None	
18/08/2015	I23	38	Black	Married	1	5	4	Neg	Neg	No	No	4	2	60	No	No	2 miscarriges,	2 ectopic
18/08/2015	I21	28	African	Married	0	2	2	Neg	Neg	No	yes	2	7	30	No	No	N/A	NO
18/08/2015	I22	31	African	Married	0	0	0	Neg	Neg	No	No	4	14	8	No	No	N/A	NO

Table 6: Patient's demographics obtained from participants at the reproductive biology clinic (continued)

Date	Sample ID	Age	Ethnicity	Marital status	Parity (Births)	Gravidity	Pregnancy losses	RPR status	HIV Status	Tampon use	New partnerlast 6 months	Vaginal sex frequency	Last intercourse(days)	Last menstruation(days)	Douching	Condom use	History of pregnancy implications	Alcohol consumption
18/08/2015	I23	38	African	Married	1	5	4	Neg	Neg	No	No	3	2	60	No	No	N/A	YES
18/08/2015	I24	36	African	Married	0	0	0	Neg	Neg	No	No	2	6	17	No	No	N/A	YES
25/08/2015	I25	24	Asian	Married	0	0	0	Neg	Neg	No	No	2	3	7	No	No	N/A	NO
25/08/2015	I26	32	African	Traditional	1	0	1	Neg	Neg	No	No	2	4	23	No	No	N/A	YES
25/08/2015	I27	24	Asian	Married	0	1	1	Neg	Neg	No	No	2	5	7	No	No	N/A	NO
25/08/2015	I28	31	African	Married	0	1	1	Neg	Neg	No	No	3	7	10	No	No	N/A	NO
01/09/2015	I29	38	White	Married	2	2	0	Neg	Neg	No	No	3	6	13	No	No	N/A	YES
01/09/2015	I30	30	African	Married	0	0	0	Neg	Neg	No	No	4	7	16	No	No	N/A	YES
01/09/2015	I31	31	African	Single	0	2	2	Neg	Neg	No	No	1	21	1	No	No	N/A	YES
01/09/2015	I32	28	African	Married	0	1	1	Neg	Neg	No	No	2	3	18	No	No	N/A	YES
08/09/2015	I33	30	Asian	Married	0	0	0	Neg	Neg	No	No	2	8	3	No	No	N/A	NO
08/09/2015	I34	27	African	Married	0	1	1	Neg	Neg	No	No	2	4	8	No	No	N/A	NO
08/09/2015	I35	37	African	Married	1	4	3	Neg	Pos	No	No	2	3	7	Yes	Yes	N/A	NO
08/09/2015	I36	31	African	Married	1	2	1	Pos	Pos	No	No	2	4	5	Yes	Yes	N/A	NO
09/09/2015	I37	32	African	Married	0	0	0	Neg	Neg	No	No	4	3	65	No	No	N/A	Yes

Table 6: Patient's demographics obtained from participants at the reproductive biology clinic (continued)

Date	Sample ID	Age	Ethnicity	Marital status	Parity (Births)	Gravidity	Pregnancy losses	RPR status	HIV Status	Tampon use	New partner last 6 months	Vaginal sex frequency	Last intercourse (days)	Last menstruation (days)	Douching	Condom use	History of pregnancy implications	Alcohol consumption
09/09/2015	I38	36	African	Married	0	2	2	Neg	Neg	No	No	2	4	26	No	No	N/A	NO
09/09/2015	I39	27	African	Married	0	2	2	Neg	Neg	No	No	3	4	6	No	No	N/A	NO
09/09/2015	I40	30	African	Married	0	1	1	Neg	Neg	No	No	2	6	9	No	No	N/A	NO
26/10/2015	I41	43	African	Married	3	5	2	Neg	Neg	No	No	1	9	4	No	No	N/A	NO
26/10/2015	I42	33	African	Married	0	0	0	Neg	Neg	No	No	2	4	10	No	No	N/A	NO
26/10/2015	I43	32	White	Married	0	0	0	Neg	neg	No	No	3	4	7	No	No	N/A	NO
27/10/2015	I44	33	African	enganged	0	0	0	Neg	Neg	No	No	2	4	4	No	No	N/A	NO
27/10/2015	I45	38	African	enganged	0	0	0	Neg	Neg	No	No	4	3	7	no	No	N/A	NO
27/10/2015	I46	29	African	Married	0	0	0	Neg	Neg	No	No	1	7	14	No	No	N/A	YES
27/10/2015	I47	30	African	Married	0	0	0	Neg	Neg	No	No	2	4	4	No	No	N/A	NO
28/10/2015	I48	34	African	enganged	0	0	0	Neg	Neg	No	No	4	3	7	No	No	N/A	NO
29/10/2015	I49	32	African	Married	0	0	0	Neg	Neg	No	No	2	4	4	No	No	N/A	NO
30/10/2015	I50	28	African	Married	0	0	0	Neg	Neg	No	No	4	3	7	No	No	N/A	NO
31/10/2015	I51	33	African	Married	0	0	0	Neg	Neg	No	No	1	7	14	No	No	N/A	YES

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

29/01/2015

Approval Certificate
New Application

Ethics Reference No.: 1/2015

Title: Detection and characterisation of mycoplasmas in women visiting an antenatal or reproductive biology clinic

Dear Mr Thabang Duba

The **New Application** as supported by documents specified in your cover letter for your research received on the 9/12/2014, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 28/01/2015.

Please note the following about your ethics approval:
- Ethics Approval is valid for 2 years.
- Please remember to use your protocol number (**1/2015**) 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.

We wish you the best with your research.

Yours sincerely

Professor Werdie (CW) Van Staden
MBChB MMed(Psych) MD FCPsych FTCL UPLM
Chairperson: Faculty of Health Sciences Research Ethics Committee

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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UNIVERSITEIT VAN PRETORIA
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Faculty of Health Sciences

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

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

30 November 2018

Approval Certificate
Annual Renewal

Ethics Reference No.: 15018475
Title: Detection and characterisation of mycoplasmas in women visiting an antenatal or reproductive biology clinic

Dear Mr TC Duba

The **Annual Renewal** as supported by documents received between 2018-11-30 and 2018-11-30 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 2018-11-21.

Please note the following about your ethics approval:

- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2019-11-30.
- Please remember to use your protocol number (15018475) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

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

We wish you the best with your research.

Yours sincerely

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

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

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Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo