The association between genital mycoplasmas and bacterial vaginosis in pregnant women with or without genital symptoms

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. I further declare that all sources cited or quoted are specified and recognised by means of an inclusive list of references.

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Who aims at excellence will be above mediocrity,
who aims at mediocrity will be far short of it

Chinese proverb

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LIST OF ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
ATCC	American Type Culture Collection
bp	Base pair
BPD	Bronchopulmonary dysplasia
BHQ	Black hole quencher
BV	Bacterial vaginosis
BVAB	Bacterial vaginosis associated bacteria
cfu	Colony forming unit
CLSI	Clinical Laboratory Standards Institute
CDC	Centres for Disease Control and Prevention
CSF	Cerebrospinal fluid
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FAM	6-carboxyfluorescein
FRT	Female reproductive tract
FVU	First void urine
g	Gram
gap	Glyceraldehyde-3-phosphate gene
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
h	Hour
H_2O_2	Hydrogen peroxide
HEX	carboxy-2', 4, 4', 5', 7, 7'-hexachlorofluoroscein
HIV	Human immunodeficiency virus
hly	Haemolysin gene
HSP	Heat shock protein
Ig	Immunoglobulin
IL	Interleukin
Kb	Kilobase
kDa	kilodalton
KOH	Potassium hydroxide
LA	Lymphoid aggregates
LBW	Low birth weight
MAFU	Maternal and Foetal Unit
Mb	Megabase
MBA	Multiple-banded antigen
MBL	Mannose-binding lectin
MgPa	*Mycoplasma genitalium* adhesin protein
MIC	Minimum inhibitory concentration
MLSKO	Macrolides, lincosamides, streptogramins, ketolides and oxazolidinones

MsrA	Methionine sulfoxide reductase
µm	Micrometre
Min	Minute
mPCR	Multiplex polymerase chain reaction
NA	Nucleic acid
NCNGU	Non-chlamydial non-gonococcal urethritis
NGU	Non-gonococcal urethritis
nt	Nucleotide
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
pH	Power of hydrogen
PID	Pelvic inflammatory disease
PFGE	Pulsed field gel electrophoresis
PROM	Premature rupture of membranes
PTB	Preterm birth
PTD	Preterm delivery
PTL	Preterm labour
qPCR	Quantitative polymerase chain reaction
RC-101	Retrocyclin-101
RNA	Ribonucleic acid
rRNA	ribosomal RNA
RTI	Reproductive tract infection
SLPI	Secretory leukocyte protease inhibitor
sp.	Species (singular)
spp.	Species (plural)
STD	Sexual transmitted disease
STI	Sexually transmitted infection
STR	Short tandem repeat
TBE	Tris-borate ethylenediaminetetraacetic acid
TLR	Toll-like receptor
Vaa	Variable adherence-associated
VDS	Vaginal discharge syndrome
VLY	Vaginolysin
WHO	World Health Organization

LIST OF BACTERIAL SPECIES

A. vaginae	*Atopobium vaginae*
G. vaginalis	*Gardnerella vaginalis*
L. acidophilus	*Lactobacillus acidophilus*
L. crispatus	*Lactobacillus crispatus*
L. gasseri	*Lactobacillus gasseri*
L. iners	*Lactobacillus iners*
L. jensenii	*Lactobacillus jensenii*
L. johnsoni	*Lactobacillus johnsoni*
L. amylolyticus	*Lactobacillus amylolyticus*
L. amylovorus	*Lactobacillus amylovorus*
L. gallinarium	*Lactobacillus gallinarium*
M. fermentans	*Mycoplasma fermentans*
M. genitalium	*Mycoplasma genitalium*
M. hominis	*Mycoplasma hominis*
S. agalactiae	*Streptococcus agalactiae*
U. parvum	*Ureaplasma parvum*
U. urealyticum	*Ureaplasma urealyticum*

LIST OF PUBLICATIONS AND CONFERENCE CONTRIBUTIONS

PUBLICATIONS

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PAPERS IN PREPARATION

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THE ASSOCIATION BETWEEN GENITAL MYCOPLASMAS AND BACTERIAL VAGINOSIS IN PREGNANT WOMEN WITH OR WITHOUT GENITAL SYMPTOMS

by

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SUMMARY

Bacterial vaginosis (BV) and genital mycoplasmas are infections of the reproductive tract that play important roles in maternal and foetal health. Genital mycoplasmas include *Mycoplasma genitalium, M. hominis, Ureaplasma parvum* and *U. urealyticum*. Infection may increase a woman's susceptibility to infection with the human immunodeficiency virus (HIV). Bacterial vaginosis associated bacteria may form biofilms that are responsible for antimicrobial resistance and about 30% of affected women will relapse within three months of treatment. Genital mycoplasmas are prone to develop point mutations, which are responsible for increased antimicrobial resistance. Infections with these bacteria become prominent during pregnancy as infection may lead to infertility and foetal death. The purpose of the study was to determine the association between genital mycoplasmas and BV in pregnant women.

Pregnant women attending the antenatal and Maternal and Foetal Unit (MAFU) clinics of a tertiary academic hospital in Pretoria, South Africa were included in the study. Self-collected vaginal swab specimens were obtained from consenting women older than 18 years of age. With the aid of microscopy, the Nugent scoring system was used to diagnose BV. Genital mycoplasmas were cultured on A2 agar and were diagnosed and speciated with a multiplex polymerase chain reaction (mPCR) assay. In addition, genital mycoplasmas were diagnosed and the antimicrobial susceptibility profiles determined with the Mycofast Revolution assay.

A quantitative real-time polymerase chain reaction (qPCR) was employed to quantify the BV associated bacteria *Atopobium vaginae* and *Gardnerella vaginalis*.

The prevalence of BV in this study was found to be 17.7% in 220 recruited pregnant women. Threshold concentrations between 10^6 to 10^7 copies/reaction of *A. vaginae* and *G. vaginalis* were found to be the best predictors of BV. Genital mycoplasmas were poorly recovered from A2 agar media, which had a contamination rate of 54.9%. An mPCR assay revealed that genital mycoplasmas were prevalent in 2.3% to 71.4% of specimens with *U. parvum* being the most prevalent species. The resistance of *Ureaplasma* species to tetracycline and erythromycin was 73% and 80%, respectively. Minor resistance to the fluoroquinolones, levofloxacin and moxifloxacin was recorded. This study found that only the genital mycoplasmas, namely *M. hominis* and *U. parvum*, were significantly associated with BV, while *M. hominis* was also significantly isolated from HIV positive women.

This study found that there is an association between BV and genital mycoplasmas. The high prevalence of BV and genital mycoplasmas suggests that current management and/or intervention strategies are insufficient. Bacterial vaginosis associated bacteria can form a polymicrobial biofilm, which confer protection against antimicrobial agents and host immune responses. These biofilms are present on genital sites like the endometrium, which is located close to the amniotic membranes, posing health risks for the pregnancy. Future research must focus on the study of *in vitro* BV biofilm models and effective treatment strategies to minimise antimicrobial resistance. In the meantime, low-cost point-of-care (POC) tests that can accurately diagnose RTIs are needed to prevent excessive and unnecessary administration of antimicrobial agents and improve maternal and foetal health in the South African health care system.

Keywords: *Atopobium vaginae*, Biofilm, Foetus, *Gardnerella vaginalis*, Genital mycoplasmas, PCR, Pregnancy, Reproductive health, Reproductive tract infections, Quantification

CHAPTER 1

Introduction

The female genital tract is a dynamic and intricate, yet balanced ecosystem that hosts a variety of different residential microflora (Diaz *et al*., 2010). The physiological changes that occur during pregnancy may disrupt this balanced ecosystem and predispose women to a potentially pathogenic microbial population (Genc and Onderdonk, 2011). Bacteria that are associated with BV and genital mycoplasmas are opportunistic bacteria that frequently form part of this pathogenic microbial population (Waites *et al*., 2009; Armstrong and Wilson, 2010). During pregnancy, infections by these bacteria pose health risks for the mother and foetus and may lead to infertility and adverse pregnancy outcomes (Guerra *et al*., 2006; Larsen and Hwang, 2010; Namba *et al*., 2010).

Bacterial vaginosis is a dysbiosis, which may or may not include genital mycoplasmas (Taylor-Robinson, 2007; Hooven *et al*., 2012). The overgrowth of and infections with these bacteria are linked to poor obstetric outcomes and increased transmission of other reproductive tract infections (RTIs) (Hooven *et al*., 2012). In South Africa, RTIs are widespread but have increased in accordance with the emergence of the Human Immunodeficiency Virus (HIV) epidemic (Rours *et al*., 2006). These RTIs increase women's susceptibility of acquiring HIV, the rates of HIV shedding and the development of Acquired Immune Deficiency Syndrome (AIDS) in HIV infected patients (Hashimoto *et al*., 2006; Woodman *et al*., 2011).

In developed countries, the use of antimicrobial agents in pregnancy is one of the main reasons for a decline in maternal and perinatal morbidity (Lockitch, 2004). The Centers for Disease Control and Prevention (CDC) (2013) recommend that all pregnant women should be screened for BV if there is any history of a preterm delivery (PTD) or a low birth weight (LBW; <2 500 g) infant, regardless of symptoms and be treated if BV positive. In addition, it is recommended that women with symptoms be screened and treated for BV (CDC, 2013). Due to difficulties related to the management of RTIs in developing countries, such as financial constraints, the World Health Organization (WHO) introduced syndromic management guidelines for treating RTIs (Johnson *et al*., 2011). The syndromic approach

involves the treatment of patients who present with a set of signs and symptoms characteristic of certain infections (Romoren *et al*., 2007; Lewis *et al*., 2008; Lewis and Maruma, 2010). Patients are treated with two or more antimicrobial agents (Romoren *et al*., 2007). In South Africa, resources are limited and no national screening programme is available for RTIs, such as BV and genital mycoplasmas. Symptomatic women, i.e. presenting with a vaginal discharge, are syndromically treated for vaginal discharge syndrome (VDS) according to the Standard Treatment Guidelines and Essential Drugs List of South Africa (National Department of Health, 2008). The regular association of genital mycoplasmas with BV is accounted for in treatment strategies by combining antimicrobial agents, which is the case for VDS (Lewis and Maruma, 2010).

A physiological discharge and the risk of candidiasis increase during pregnancy (Romoren *et al*., 2007; Omole-Ohonsi and Nwokedi, 2011). This makes symptoms like vaginal discharge and lower abdominal pain even less specific for BV and genital mycoplasma infections (Romoren *et al*., 2007). Infections by these bacteria may be asymptomatic in the majority of cases (Sung *et al*., 2006; Leitich and Kiss, 2007). Johnson *et al*. (2011) studied the effect of syndromic management interventions on the prevalence of RTIs in South Africa. The prevalence of symptomatic RTIs was shown to be reduced by syndromic management approaches but had little influence on the prevalence of RTIs that typically present with no symptoms (Johnson *et al*. 2011). This poses a major problem as many of these infections may not be detected and left untreated. Leitich and Kiss (2007) performed a meta-analysis in 2003, which was expanded in 2006 to include a total of 32 studies and 30 518 patients. The analysis comprised studies of women <37 weeks' gestation who were screened for BV either with clinical criteria or criteria based on Gram-stain findings (Leitich and Kiss, 2007). The authors reported that BV more than doubled the risk of preterm birth (PTB; i.e. birth at 22 to 36 weeks gestation) in asymptomatic patients (Leitich and Kiss, 2007). Other studies in developing countries (Hylton-Kong *et al*., 2004; Tann *et al*., 2006; Clark *et al*., 2009) indicated low correlations between infections and symptoms, which limits the effectiveness of syndromic management.

The HIV prevalence among pregnant women (aged 15 to 49 years) in South Africa is estimated to be 29.5% (National Department of Health, 2011). Syndromic management may be a cost-effective approach of reducing HIV infection rates (White *et al*., 2008). However,

the failure of the syndromic approach to decrease the prevalence of asymptomatic RTIs in South Africa necessitates the investigation of enhanced diagnostic and treatment strategies. This may aid in reducing the stress placed on an already overburdened health system (Hontelez *et al*., 2011).

Aim

The purpose of this study was to determine the association between *M. genitalium, M. hominis*, *U. parvum* and *U. urealyticum* and bacterial vaginosis in pregnant women, with and without confirmed bacterial vaginosis, attending a tertiary academic hospital in Pretoria, South Africa.

Objectives

1. To obtain (self-collected) vaginal swabs from 200 pregnant women
2. To diagnose bacterial vaginosis using the Nugent scoring system
3. To culture *Ureaplasma* spp. and *M. hominis*
4. To extract bacterial DNA
5. To perform a multiplex PCR for the detection of *M. genitalium, M. hominis*, *U. parvum* and *U. urealyticum*
6. To quantify *A. vaginae* and *G. vaginalis* with real-time PCR
7. To perform data analysis

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CHAPTER 2

Literature review

2.1 Introduction

Bacterial vaginosis and genital mycoplasmas are associated with several infections and adverse pregnancy outcomes, such as urethritis, pelvic inflammatory disease (PID), PTB, premature rupture of membranes (PROM) and miscarriage in affected women (Diaz *et al*., 2010; Xiao *et al*., 2010). Mycoplasmas are vertically transmitted and colonisation has been associated with chronic lung diseases in children (Waites *et al*., 2005; Larsen and Hwang, 2010). Bacterial vaginosis and genital mycoplasma infections are often elusive or sub-clinical in nature and it is not always possible to prove the pathogenic nature of these bacteria (Sung *et al*., 2006).

Several critical reproductive tract infection/sexually transmitted infection (RTI/STI)-related complications are theoretically easy to manage and inexpensive to prevent, yet these complications continue to contribute to high morbidity and mortality rates of mothers and their babies (WHO, 2005). The main reasons for the slow progress in BV and mycoplasma management may be due to (i) low and inadequate screening rates, (ii) flawed diagnostic techniques and (iii) insufficient treatment coverage for the bacteria present in BV positive cases (Hogan *et al*., 2007).

There is no routine screening for BV and genital mycoplasmas in the South African public health sector and therefore the prevalence and antimicrobial susceptibility patterns need to be monitored on a regular basis (Govender *et al*., 2009). The gold standard for the diagnosis of genital mycoplasmas is culture, which has a sensitivity of 60% in expert laboratories (Waites *et al*., 2012). Bacterial vaginosis is diagnosed by two gold standard methods, the Nugent scoring system and Amsel's criteria; however, these two methods often do not agree (Menard *et al*., 2010). The diagnosis of BV is further complicated by the fact that different morphotypes of bacteria are present during the different trimesters of pregnancy (Waters *et al*., 2008). Bacterial vaginosis is a polymicrobial condition and although current regimens for BV treatment are efficient, about 15% to 30% of treated women will relapse within three months, a condition known as recurrent bacterial vaginosis (Hay, 2000). There is increased

resistance of genital mycoplasmas to macrolide antimicrobial agents, the agents frequently used to treat these infections during pregnancy (Dongya *et al*., 2008; Xiao *et al*., 2010).

2.2 The vaginal ecosystem

The human vagina is an extremely dynamic, nutrient-rich cavity for bacteria that develop into a unique microbiota (Mirmonsef *et al*., 2011). It has been described as an intricate and diverse ecosystem, which determines vaginal health (Diaz *et al*., 2010; Danielsson *et al*., 2011). This bionetwork mainly comprises a wide spectrum of aerobic and anaerobic bacterial genera and species in healthy asymptomatic women, with the *Lactobacillus* genus dominating (Donati *et al*., 2010). The vagina of healthy women contains 10^9 bacterial colony forming units per gram (cfu/g) of vaginal fluid and is an ecosystem in equilibrium (Verhelst *et al*., 2005). The genital tract of women as a whole consists of different parts, each hosting different types of microbes (Lamont *et al*., 2011). The cervix, the upper vagina and the lower vagina differ in their composition of microbial flora (Lamont *et al*., 2011). Endogenous and exogenous influences, such as pregnancy, the host's age, state of health and geographical variation may allow the composition of the vaginal ecosystem to transform over time (Kiss *et al*., 2007; Witkin *et al*., 2007).

2.2.1 The female genital tract

The genital tract of women consists of a series of interconnected cavities (Witkin *et al*., 2007). A canal is formed *via* this interconnection: The vulva (external genitalia) leads into the vagina that is joined in sequence to the ectocervix, the endocervix and the uterus and then to the Fallopian tubes (Witkin *et al*., 2007). This canal enables the delivery of spermatozoa to the internal cavities and the movement of the menses and mature foetus to the exterior (Witkin *et al*., 2007).

The upper female reproductive tract (FRT) comprises the Fallopian tubes, the uterus and the endocervix and is covered by a single layer of columnar epithelial cells joined by tight junctions (WHO, 2005; Hickey *et al*., 2011). These epithelial cells are covered with a protective mucous layer and underlying the epithelial cells are innate and adaptive immune cells and lymphoid aggregates (LA) (Hickey *et al*., 2011) (Figure 2.1).

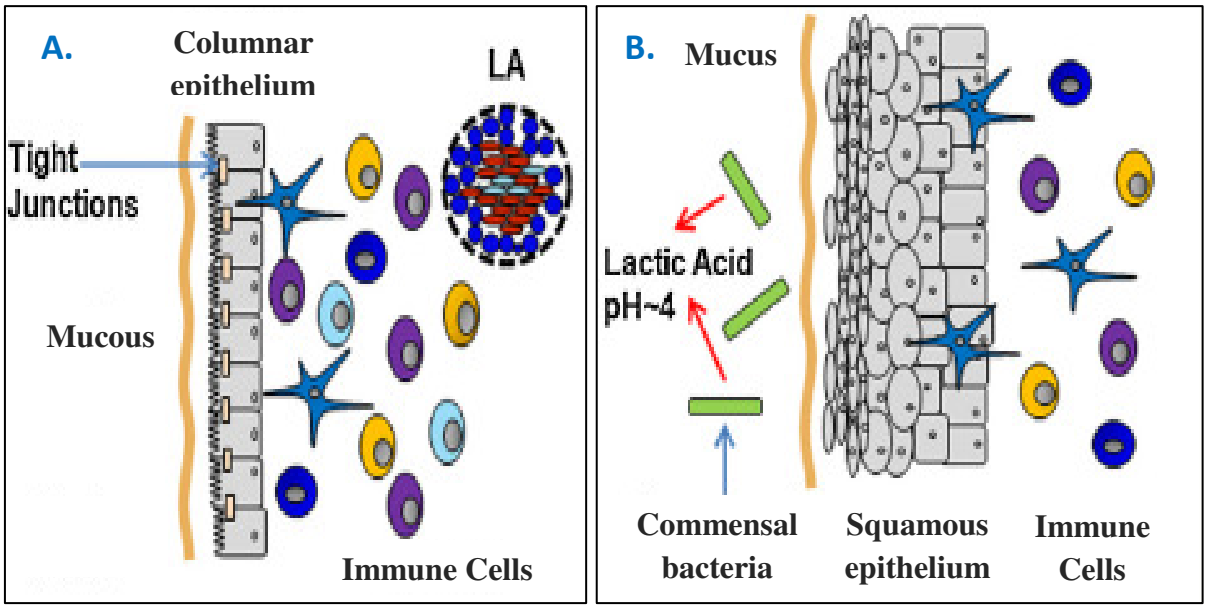

Figure 2.1: A representation of the internal (A) upper and (B) lower FRT. Both the upper and lower FRTs' epithelial cells are covered by a protective mucous layer and below this epithelial layer immune cells are present (Hickey *et al.*, 2011)

The lower FRT consists of the ectocervix and the vagina and is lined with stratified epithelial cells and is, comparable to the upper FRT, overlaid by a protective mucous layer (WHO, 2005; Hickey *et al.*, 2011). The bacterial population, which inhabits the lower FRT, maintains an acidic vaginal pH (Hickey *et al.*, 2011).

2.2.2 Normal vaginal flora dominated by *Lactobacillus* species

Lactobacilli, especially hydrogen peroxide (H_2O_2)-producing strains, are the most eminent markers of normal flora and are important indicators of a healthy vaginal milieu in menarchal women (Donders, 2007; Genc and Onderdonk, 2011). Prior to the advent of molecular methods, *Lactobacillus acidophilus* was believed to be the main vaginal species in healthy women of childbearing age (Kiss *et al.*, 2007). Although numerous species have been identified in the vagina, the most frequent ones include the homofermentative *L. crispatus, L. iners, L. gasseri* and *L. jensenii* with differences in species and relative species numbers (Donati *et al.*, 2010). These bacterial species manifest themselves in the vagina by attaching to glycolipid receptors of the epithelia *via* pili that act as ligands (Danielsson *et al.*, 2011).

Lactobacilli not only establish a healthy vagina with high numbers, but also by maintaining an acidic environment (pH 3.8 to 4.5) with lactic acid production through glycogen metabolism

(Danielsson *et al*., 2011; Lamont *et al*., 2011). These bacteria can kill off and prevent the proliferation of other bacteria by the production of bacteriocins, antibiotic toxic hydroxyl radicals, H_2O_2 and probiotics and by blocking attachment to the vaginal epithelium (Donders, 2007; Zodzika *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 (Danielsson *et al*., 2011; Zodzika *et al*., 2011).

2.2.3 Normal vaginal flora dominated by bacteria other than lactobacilli

In some healthy women (ranging from 7% to 33%), a dynamic vaginal ecosystem is still maintained where bacterial species other than lactobacilli fill the niche (Witkin *et al*., 2007). Comparable to the lactobacilli, *Atopobium*, *Leptotrichia* and *Megasphaera* produce lactic acid and are able to retain a normal (moderately acidic) vaginal pH (Donati *et al*., 2010; Linhares *et al*., 2011). Non-*Lactobacillus* bacteria take part in mixed acid fermentation where other organic acids, such as mydriatic, linoleic and acetic acid are typically produced along with lactic acid (Linhares *et al*., 2011). Consequently, the presence of potentially pathogenic microbes, such as *Escherichia coli, Gardnerella vaginalis, Mycoplasma* spp., *Peptostreptococcus, Prevotella, Pseudomonas,* group B *Streptococcus* (GBS; *S. agalactiae*) and *Ureaplasma* spp. does not represent an anomalous state (Genc and Onderdonk, 2011; Lamont *et al*., 2011). These bacteria may be present in relatively low numbers and concentrations under the normal acidic conditions (≤ pH 4.5) of the vagina and do not cause any apparent (communicable) complications (Genc and Onderdonk, 2011). Colonisation of genital mycoplasmas (more commonly *Ureaplasma* spp. and/or *M. hominis*) occurs more frequently in young girls rather than boys and is mostly due to cervical or vaginal contact during birth (Taylor-Robinson, 2007). Colonisation in children exposed to these bacteria during birth tends not to persist and the colonisation after puberty is largely due to sexual contact (Taylor-Robinson, 2007).

2.2.4 Vaginal pH and microbial composition

The vaginal pH fluctuates significantly over a woman's life span and is directly related to the microbial composition of the vagina (Donati *et al*., 2010; Genc and Onderdonk, 2011). However, a variable pH is the collective result of processes and products that involve lactobacilli, oestrogen and glycogen (Danielsson *et al*., 2011). In a new-born, where there is no apparent colonisation of any microbes just yet, the vaginal pH is approximately 5 and only

increases to the neutral range (≥7) by 6 weeks of age (Linhares *et al*., 2011). A pH of 5 is maintained in premenarchal girls where aerobic and anaerobic cocci and rods, equivalent to that of the periurethral area, dominate (Danielsson *et al*., 2011). As girls grow older and reach their menarche, the microbiota steadily changes as oestrogen levels start to rise with ovary function (Danielsson *et al*., 2011).

Glucose is stored in the vaginal mucosal cells as glycogen (Linhares *et al*., 2011) and the vaginal epithelium will thicken as glycogen accumulates (Danielsson *et al*., 2011). When cells need energy, the glycogen is converted back to glucose, which under anaerobic conditions, is eventually metabolised to lactate, the conjugate base of lactic acid (Linhares *et al*., 2011). This endogenous degradation of glycogen will result in a pH of nearly 5 (Danielsson *et al*., 2011). By means of diffusion, lactic acid will be dispersed into the extracellular environment and accumulate (Linhares *et al*., 2011). Rectal lactobacilli can reach amounts of up to 10^7 to 10^8 per gram vaginal fluid, since growth is supported by the elevated concentration and low pH of glycogen in the stratified vaginal epithelium (Danielsson *et al*., 2011). In reproductive-age women the final pH ranges from 3.8 to 4.4 and is established by both the lactic acid secreted by epithelial cells and acid production by the endogenous microflora (Linhares *et al*., 2011).

2.3 Changes in pregnancy

The microbiological fluctuations that typically occur during the course of the menstrual cycle are suspended when females fall pregnant (Genc and Onderdonk, 2011). A healthy vaginal microbial flora may be maintained; however, if the composition of this microflora changes in an unpredictable fashion it may disrupt the normal gestational events and lead to pregnancy complications (Genc and Onderdonk, 2011).

During pregnancy, oestrogen levels are elevated and glycogen synthesis is increased (Lin *et al*., 2011). Lactobacillary activity and proliferation are favoured by the increased glycogen available, which leads to an enhanced epithelial tropism (Donati *et al*., 2010). The resident vaginal flora, especially the activity of *Lactobacillus*, is believed to protect pregnant women against FRT infections (Donati *et al*., 2010; Lamont *et al*., 2011). However, during pregnancy there is a typical vaginal discharge because of increased levels of serum oestrogen (Omole-Ohonsi and Nwokedi, 2011). This discharge will be heavier and contain more

cervical mucous as the pregnancy progresses and predisposes women to RTIs/STIs (Omole-Ohonsi and Nwokedi, 2011).

2.3.1 Infections of the female reproductive tract

Reproductive tract infections are infections of the human genital tract that are categorised according to the mode of acquisition (WHO, 2005). Different infectious agents establish themselves in different sites of the female reproductive organs (WHO, 2005) (Figure 2.2).

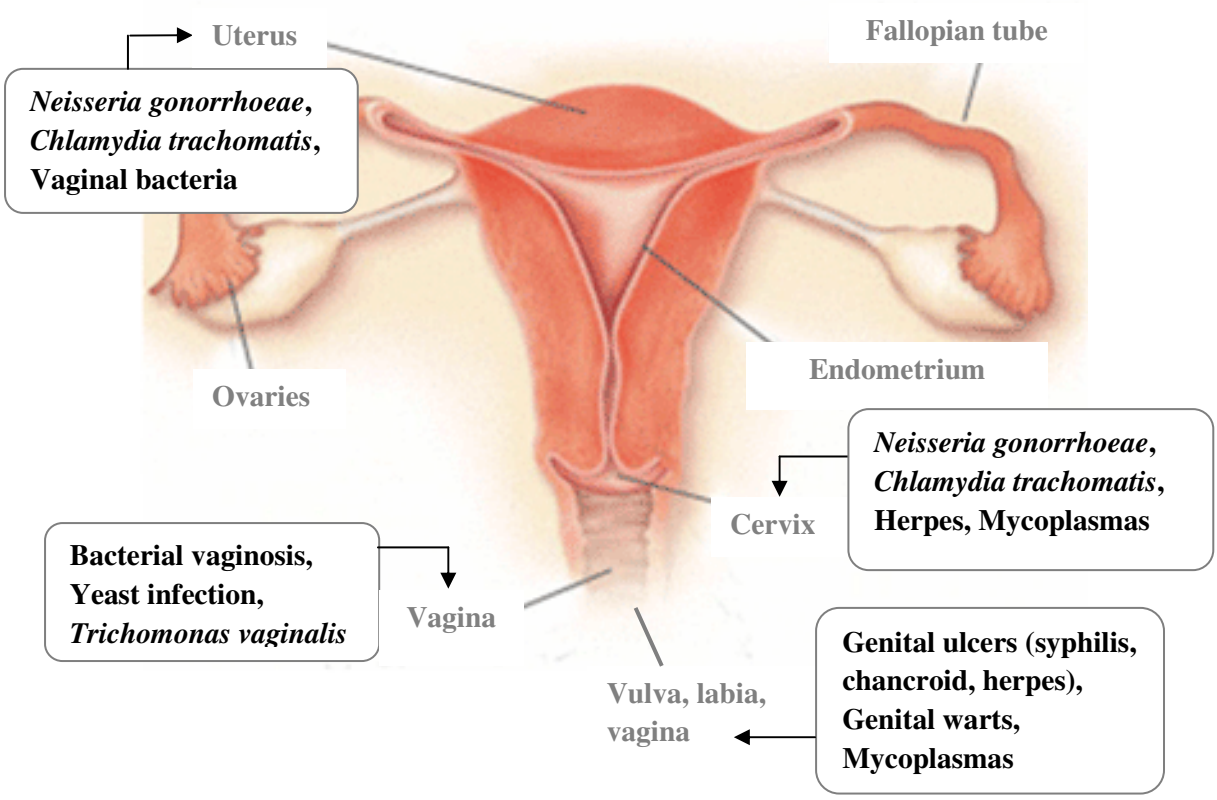

Figure 2.2: The female reproductive organs that generally serve as sites of infection and possible infectious agents/diseases (WHO, 2005; Welch, 2005; South Florida Woman's Health Associates Inc., http://www.sfwha.com/MIGS/2.htm)

While some RTIs like gonorrhoea and genital mycoplasma infections are sexually transmitted (Imudia *et al*., 2008), others like BV and yeast infections are classified as endogenous infections, since these infections are caused by the overgrowth of microorganisms commonly found in the vagina (WHO, 2005). Pregnant women with RTIs can have complications during the gestation period, which may result in adverse pregnancy outcomes (WHO, 2005).

2.3.2 Vaginal immunity

Infection by potentially pathogenic microorganisms is not only prevented by the activity of the normal vaginal microflora, but also by a finely tuned innate and adaptive immune response (Witkin *et al*., 2007; Danielsson *et al*., 2011). The vaginal mucosa is the primary point of interaction between microorganisms and the host's genital tract and the innate immune responses at this epithelial lining play an integral role against microorganism invasion (Witkin *et al*., 2007; Genc and Onderdonk, 2011). The innate immune system identifies microbial intruders instantly *via* the pathogen-associated molecular patterns (PAMPs), while the adaptive immune system produces cell-mediated and antibody-mediated immunity, which are antigen-specific (Witkin *et al*., 2007). Innate immune system components functioning in the vagina may include membrane-bound Toll-like receptors (TLR), surfactant protein A, lactoferrin, complement component, β-defensins, secretory leukocyte protease inhibitor (SLPI), mannose-binding lectin (MBL), heat shock proteins and nitric oxide (Witkin *et al*., 2007; Linhares *et al*., 2011). The antigen-specific B lymphocytes, which are predominantly present in the endocervix and vagina of the female lower genital tract, produce IgG and IgA antibodies locally that are secreted into the mucosa (Witkin *et al*., 2007; Hickey *et al*., 2011).

2.4 Bacterial vaginosis

Bacterial vaginosis is considered an endogenous RTI that is increasingly linked with adverse perinatal sequelae (Donati *et al*., 2010). It is a polymicrobial condition that is dubbed a sexually associated condition instead of a sexually transmitted one because it is associated with sexual activity; however, sexual activity is not the sole determinant for its occurrence (Morris *et al*., 2001; Verstraelen *et al*., 2010). The aetiology of BV is poorly understood and more accurate and standard diagnostic markers need to be defined (Schoeman, 2002; Danielsson *et al*., 2011).

2.4.1 History of bacterial vaginosis

Albert Döderlein documented in 1892 that culturable organisms, called Döderlein's bacilli, supply lactic acid, which prevents the growth of potential pathogens (Lamont *et al*., 2011). *Lactobacillus* spp. were shown to be present in the vaginal fluids of healthy women but lacking in women with postpartum endometritis, while anaerobic cocci and *Mobiluncus* spp. were linked with postpartum endometritis and vaginal discharge (Schoeman, 2002; Donders,

2007). However, in 1921 the vaginal flora was more formally characterised as either most likely pathogenic, intermediate and least likely pathogenic based on Gram staining reactions of vaginal fluid (Schoeman, 2002). In 1928, Stanley Thomas collectively grouped Döderlein's bacilli as *Lactobacillus acidophilus*. This species was later, in 1980, shown to be heterogeneous and referred to as the *L. acidophilus* complex, including the species *L. acidophilus, L. amylolyticus, L. amylovorus, L. crispatus, L. gallinarium, L. gasseri, L. iners, L. jensenii* and *L. johnsoni* (Lamont *et al*., 2011).

In 1950, a condition known as non-specific vaginitis with unknown aetiology was associated with the absence of lactobacilli and the presence of *Bacteroides* spp. (Schoeman, 2002). Non-specific vaginitis was later renamed as bacterial vaginosis as it became clear that there is no obvious clinical inflammation or increased levels of vaginal leukocytes in BV positive women (Danielsson *et al*., 2011). Around 1954 and 1955, Gardner and Dukes attempted to describe a sole bacterium responsible for BV, first called *Haemophilus vaginalis* and later renamed as *Corynebacterium vaginale* (Danielsson *et al*., 2011). However, the distinctive characteristics of the newly described bacterium resulted in the formation of a whole new genus, *Gardnerella* (Donders, 2007). Even though *G. vaginalis* was practically 100% prevalent in BV positive women, it was also found to be present in ± 50% of healthy women (Schoeman, 2002).

2.4.2 Definition and classification of bacterial vaginosis

Bacterial vaginosis is an ecological disorder in which the presiding *Lactobacillus* spp. of the vagina is disturbed and replaced by elevated concentrations of endogenous aerobes and anaerobes, causing the pH to rise from less than 4.5 to 7.0 or more in affected women (Verhelst *et al*., 2005; Donders, 2007). Responsible bacteria frequently include *A. vaginae, Bacteroides* spp., *Fusobacterium* spp., *G. vaginalis*, *Mobiluncus* spp., *Mycoplasma hominis*, *Peptostreptococcus* spp., *Prevotella* spp., and *Ureaplasma* spp. (Cauci, 2004; Genc and Onderdonk, 2011). These potentially pathogenic bacteria may overgrow and lead to BV when bacterial numbers increase uncontrollably to reach 100- to 1000 fold the normal vaginal levels (Turovskiy *et al*., 2011).

Despite the attempts of Gardner and Dukes to prove that *G. vaginalis* is solely responsible for BV, it is never found as the only bacterium in BV patients (Srinivasan and Fredricks, 2008).

Molecular studies revealed that the collection of bacteria related to BV can differ substantially between individuals (Fredricks *et al*., 2009). Novel bacterial species, which are highly specific for BV, have been identified (Fredricks *et al*., 2005; Haggerty *et al*., 2009). Additional to *A. vaginae*, these bacterial species include *Leptotrichia/Sneathia* spp., bacteria closely related to *Megasphaera* spp. and three bacteria of the order *Clostridiales*, including BV-associated bacteria (BVAB) types 1, 2 and 3 (Fredricks *et al*., 2005; Haggerty *et al*., 2009).

2.4.3 Epidemiology of bacterial vaginosis

The prevalence of BV ranges from 8% to more than 40% in reproductive age women in developed countries (Hay, 2010; Rampersaud *et al*., 2012). In African nations, the prevalence rates of BV have been shown to range from 18% to 70% (Atashili *et al*., 2008) and Denslow *et al*. (2011) found a prevalence of 54% among 1 954 HIV-seropositive South African women in Johannesburg. Bacterial vaginosis occurs in approximately 20% of pregnant women (Donati *et al*., 2010). Waters *et al*. (2008) reported that BV is the most prevalent in the first trimester of pregnancy but less prevalent in the second and third trimesters. Ethnicity is an imperative determining factor in vaginal colonisation by various bacteria and women of African ethnicity are at an increased risk of developing BV (Hay, 2010). A survey from 2001 to 2004 revealed that the prevalence of BV among African-Americans was 3.31 times higher than among Caucasians (Livengood, 2009; Klatt *et al*., 2010). This can partially be elucidated by host genetics that play a role in the occurrence of BV; however, the apparent reasons still remain unclear (Danielsson *et al*., 2011). A study by Yen *et al.* (2003) found that oral contraceptive use provided some inexplicable protective effect in the development of BV.

A shift from normal vaginal flora to one indicative of BV does not necessarily result in symptoms (Lamont *et al*., 2011). Many BV cases are either paucisymptomatic or completely asymptomatic (± 50% of cases) (Livengood, 2009; Donati *et al*., 2010). However, when a patient is BV positive; clinical symptoms generally include a thin, grey, malodorous (fishy) discharge that may include local irritation (Srinivasan *et al*., 2009). The fishy smell is the result of amines (cadaverine, putrescine and trimethylamine) produced by the anaerobes present (Livengood, 2009). These symptoms are aggravated when the vaginal pH increases, for instance during menses and after sex (Livengood, 2009). At about the time of menses, BV can occur spontaneously and may resolve spontaneously in mid-cycle (Hay, 2010).

Although it is widely accepted and reported that BV is not sexually transmitted (male-to-female and female-to-male heterosexual transmission), this issue is somewhat controversial (Hay, 2010). Bacterial vaginosis can be sexually transmitted in women who have sex with women (Livengood, 2009; Danielsson *et al*., 2011). This is supported by the Gardner and Dukes study where healthy young women who were inoculated with the fluid of BV positive women, had resulting symptoms characteristic of BV (Srinivasan *et al*., 2009). However, BV has been reported in 18% of sexually inexperienced women (Yen *et al.*, 2003).

The carriage of *G. vaginalis* in the urethra and prepuce of males has been reported (Verstraelen *et al*., 2010). Schwebke (2009) established that carriage is directly associated with condom use. This strongly supports the theory of female-to-male transmission of *G. vaginalis* and other BV-associated bacteria instead of the opposite (Verstraelen *et al*., 2010). Suspected male-to-female transmission of BV by male partners who are colonised with *G. vaginalis* is not excluded (Verstraelen *et al*., 2010). Sexually active and heterosexual males are significantly more colonised than prepubertal boys and homosexual men, respectively (Verstraelen *et al*., 2010). The concept of male-to-female heterosexual transmission is opposed by (i) the treatment of male partners that is not beneficial as it does not result in a decline in BV prevalence (Verstraelen *et al*., 2010) and (ii) the fact that there is no solitary aetiological agent responsible for BV (Turovskiy *et al*., 2011).

2.4.4 Predisposing and risk factors for bacterial vaginosis

The disruption of normal vaginal homeostasis or the induction of local inflammatory responses, by previous or existing viral and bacterial infections, increases the susceptibility for the acquisition of BV (Cherpes *et al*., 2008; Danielsson *et al*., 2011). The loss of H_2O_2-producing lactobacilli increases the pH of the vaginal environment, which in its turn increases the likelihood of BV acquisition (Cherpes *et al*., 2008). The disturbance of the vaginal flora may be mediated by hormonal status, foreign bodies, vaginal blood and/or physiological or non-physiological changes (Tibaldi *et al*., 2009). The alkaline buffering action of semen nullifies the acidity of the vagina for several hours after intercourse and it is likely that this brief loss of acidity is permissive for anaerobic bacterial overgrowth (Cherpes *et al*., 2008). Important risk factors include: (i) concurrent use of medications, (ii) low socioeconomic status, (iii) increasing age, (iv) cigarette smoking, (v) young age of coitarche, (vi) precarious

practices, such as vaginal douching, (vii) the use of intrauterine devices, (viii) a new sexual partner and (ix) multiple sexual partners (CDC, 2010; Zodzika *et al*., 2011).

2.4.5 Immune system components and responses in bacterial vaginosis

Changes in innate immunity are partially liable for triggering the conversion of a vaginal microflora controlled by lactobacilli to one that resembles BV (Genc and Onderdonk, 2011). The suggested mechanisms may include: (i) the insufficient release and/or function of mannose-binding lectin, (ii) reduced TLR activation, (iii) amplified production of extracellular heat shock protein 70 (Hsp70) as well as (iv) the reduction in vaginal SLPI (Genc and Onderdonk, 2011). These factors may lead to the disruption of controlled inflammation that inhibits the overgrowth of microorganisms in the vagina (Koumans *et al*., 2007; Genc and Onderdonk, 2011).

A characteristic of BV is the absence of inflammation as there is no increase in the number of circulating leukocytes; there is a very low production of interleukin 8 and a slight rise in interleukin 1 levels (Donati *et al*., 2010). Nevertheless, a subgroup of women produces a local pro-inflammatory response (Genc and Onderdonk, 2011). Toll-like receptors transduce an inflammatory signal in cells upon recognition of microbial products (Genc and Onderdonk, 2011), which leads to the production of pro-inflammatory cytokines and induction of the adaptive immune response (Witkin *et al*., 2007). Pregnant women with BV and women who are heavily colonised with *G. vaginalis* and anaerobic Gram-negative rods, contain elevated levels of pro-inflammatory cytokines and are at an increased risk for PTB (Genc and Onderdonk, 2011). Genital mycoplasmas, *Prevotella* spp. and *Bacteroides* spp. are all microorganisms associated with PTB (Genc and Onderdonk, 2011).

2.4.6 Pathogenesis and microbial structure of bacterial vaginosis

The pathogenesis of BV is poorly understood and defined. There are at least two suggested models, which explain the possible pathogenesis of BV and which are both supported by literature (Figure 2.3) (Srinivasan and Fredricks, 2008). The *Lactobacillus* depletion model proposes that there is an initial reduction in H_2O_2-producing lactobacilli, allowing the overgrowth of facultative anaerobes, which results in BV (Srinivasan and Fredricks, 2008). The primary pathogen model proposes that the entry of facultative anaerobes causes the displacement of lactobacilli, thereby resulting in BV (Srinivasan and Fredricks, 2008).

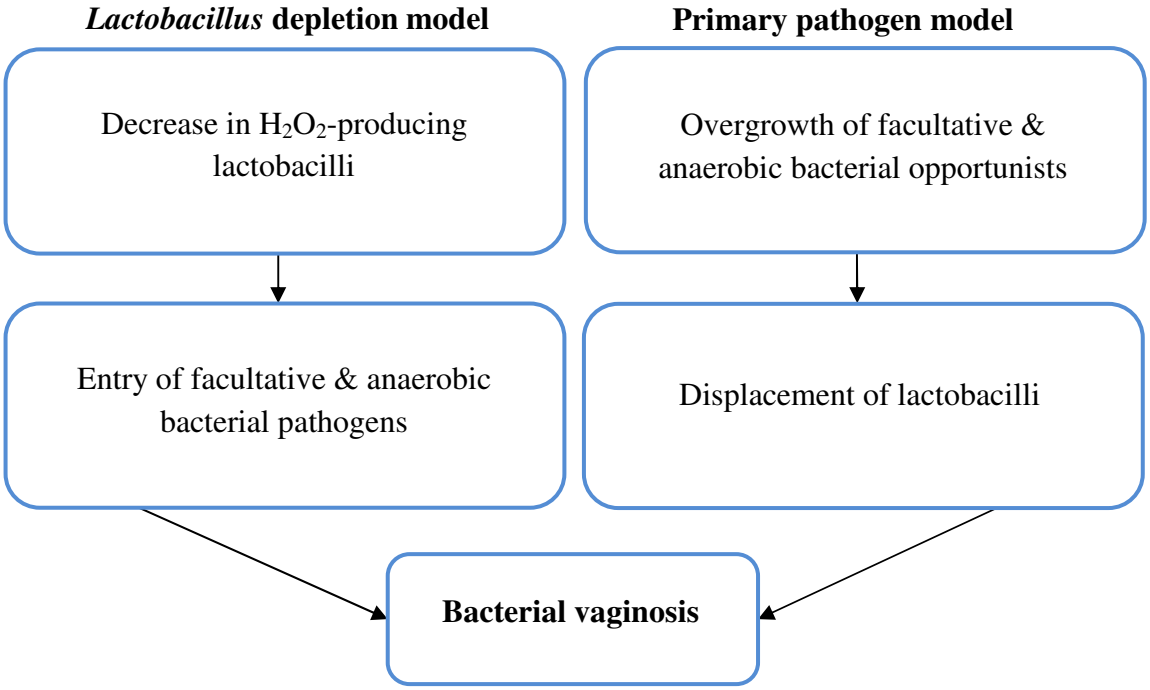

Figure 2.3: The *Lactobacillus* depletion and the Primary pathogen models that elucidate the possible pathogenesis of bacterial vaginosis (Srinivasan and Fredricks, 2008)

The *Lactobacillus* depletion model is supported by the notion that a rise in the vaginal pH occurs first with subsequent anaerobic bacterial overgrowth (Kiss *et al*., 2007; Cherpes *et al*., 2008). The levels of *A. vaginae* in the vagina have been found to be strongly associated with vaginal pH levels (Marconi *et al*., 2012). It has been suggested that strains of *A. vaginae* have the potential to produce varying amounts of lactic acid but not to such an extent to protect the normal vaginal flora (Marconi *et al*., 2012). Udayalaxmi *et al*. (2012) argued that the rise in vaginal pH occurs much later in the condition. The latter argument supports the primary pathogen model as it is postulated that BV bacteria first adhere to the vaginal epithelium, proliferate and then create a dense biofilm (Udayalaxmi *et al*., 2012). A biofilm is an intricate collection of sessile bacterial cells, which is covered by an extracellular matrix of biopolymeric substances (García-Castillo *et al*., 2008). The biofilm is by no means affected by the increase in pH, which may be the result of metabolic events of the amplified bacterial population (Udayalaxmi *et al*., 2012).

The work done and suggestions made by Swidsinski *et al*. (2010) raises new questions and further complicates the pathogenesis of BV. It is documented that *Gardnerella* can be present

in two forms: cohesive and dispersed (Swidsinski *et al*., 2010). Cohesive *Gardnerella* was shown to appear as brickwork-like structures, characteristic of biofilms and is most concentrated on epithelial cells (Swidsinski *et al*., 2010). *Atopobium vaginae* was present in low numbers in the biofilm network (Swidsinski *et al*., 2010). Dispersed *Gardnerella* surrounds leukocytes instead of epithelial cells and is occasionally concentrated to small clusters of 10 to 20 bacteria (Swidsinski *et al*., 2010). Swidsinski and colleagues (2010) found cohesive *Gardnerella* to be present in all BV positive patients and their partners and was sexually linked as opposed to dispersed *Gardnerella*, which was not (Swidsinski *et al*., 2010). It is possible that each of the two forms of *Gardnerella* may be responsible for a different pathogenesis model (Swidsinski *et al*., 2010). The authors inferred that *Gardnerella* biofilms is an entity different from BV and should be renamed gardnerellosis with the responsible *Gardnerella* strains renamed to *Gardnerella genitalis* (Swidsinski *et al*., 2010).

The formation of biofilms may be due to certain properties, which some *Gardnerella* strains possess, such as pathogenicity islands, virulence factors and plasmids or it may be a polymicrobial synergism between bacteria, e.g. *Gardnerella* and *Atopobium* spp. (Swidsinski *et al*., 2010). The pathogenic function of a biofilm is to allow the bacteria to repel the host's immune defences and tolerate higher concentrations of antimicrobial agents, explaining the recurrence rates of BV (Danielsson *et al*., 2011). High vaginal concentrations of *G. vaginalis* and *A. vaginae* indicate that these two species are most strongly associated with BV (De Backer *et al*., 2006; Menard *et al*., 2012). A study by Bradshaw *et al*. (2006) supports a synergism between *G. vaginalis* and *A. vaginae*. Several researchers suggested that infection with *A. vaginae* is even more specific (and a diagnostically more valuable marker) for BV than infection with *G. vaginalis* (Bradshaw *et al*., 2006; Trama *et al*., 2008). These two species are strongly associated with bacterial biofilm (Swidsinski *et al*., 2005). Bacterial vaginosis-associated bacteria one (BVAB1) have been found to adhere to vaginal epithelial cells similar to clue cells (Fredricks *et al*., 2005). Clue cells are epithelial cells covered with Gram-variable pleomorphic rods and are desquamated cells from a biofilm (Swidsinski *et al*., 2005). The diverse findings emphasise the polymicrobial nature of BV and its pathogenesis.

Despite the lack of a definitive pathogenesis model, several components have been identified to act as virulence factors. Some *G. vaginalis* strains have anti-IgA activity and similar to sialidases and cleavage enzymes produced by some bacteria, attenuate the defensive action of

G. vaginalis-specific IgA (Cauci, 2004; Donders, 2007). Additional bacterial enzymes believed to play a role in the pathogenesis of BV include proteases, *G. vaginalis* haemolysins and mucinases (Donders, 2007). Mucinase and sialidase, two hydrolytic enzymes, may enhance placental inflammation and weakening of the chorioamniotic membrane (Nelson *et al*., 2007; Cauci *et al*., 2008). These enzymes may promote increased ascending of lower genital tract organisms and sialidase increases the probability of PTB (Cauci, 2004). Protease activity may lead to intrauterine death by stimulating the production of pro-inflammatory cytokines and PROM and/or PTL by stimulating phospholipase A2 production (Govender *et al*., 1996; Nelson *et al*., 2007). Vaginolysin (VLY), a human-specific cytolysin produced by *G. vaginalis*, is responsible for lysing erythrocytes and epithelial cells at higher pH levels (Hooven *et al*., 2012). The ability of different *Lactobacillus* species to produce varying amounts of H_2O_2 is said to have potential effects on pregnancy outcome (Onderdonk *et al*., 2003; Wilks *et al*., 2004).

2.4.7 Bacterial vaginosis and HIV

The composition of the female reproductive tract makes women two times more likely than men to acquire HIV (Turovskiy *et al*., 2011). The resulting imbalanced vaginal flora associated with BV, the replacement of lactobacilli and the subsequent rise in pH create a more permissive milieu for HIV acquisition and proliferation (Sha *et al*., 2005; Turovskiy *et al*., 2011). Bacterial vaginosis enhances viral replication and BV-associated bacteria directly upregulate the replication of HIV through a heat-stable HIV-inducing factor (Zariffard *et al*., 2005; Johnson and Lewis, 2008; Verstraelen *et al*., 2010). Vaginal shedding of HIV is propagated by BV and women who are affected by both HIV and BV can shed virus particles up to six times more versus those who are BV negative (Verstraelen *et al*., 2010; Danielsson *et al*., 2011; Lamont *at el*., 2011). Bacterial vaginosis can act as a co-factor for HIV and conversion to seropositivity (Lamont *et al*., 2011).

2.4.8 Clinical manifestations associated with bacterial vaginosis

Women with PID are more commonly affected by BV but this disease entity alone does not result in pruritus, dysuria, burning or any inflammation in the vagina (Klebanoff *et al*., 2004; Sobel *et al*., 2012). Nevertheless, BV plays an antagonistic role in pregnancy as it favours ascending infections (from the vagina to the chorioamnion) and is consequently considered a risk factor for poor obstetric outcomes (Guerra *et al*., 2006). Bacterial vaginosis-associated

bacteria and the toxins produced by these bacteria can cross the placenta and result in foetal brain damage (Turovskiy *et al*., 2011).

The results of the meta-analysis by Leitich and Kiss (2007) confirmed that BV is associated with PTB and late miscarriage. The first 16 weeks of pregnancy possibly marks a critical stage during which BV enters the upper genital tract because women in this gestation period are at highest risk for PTB (Guaschino *et al*., 2006). Theoretically, the inception of PTL related to BV is due to its potential to cause inflammation of the choriodecidual space and activating pathways of labour (Denney and Culhane, 2009). Bacterial vaginosis is frequently reported in women with premature rupture of membranes, chorioamnionitis, LBW, postpartum endometritis and postoperative abortive infections (Guerra *et al*., 2006; Denney and Culhane, 2009).

The reason why some women with BV are more prone to deliver preterm can in part be explained by genotype-environment interactions (Denney and Culhane, 2009). The hypothesis is that only women who have a genetic predisposition to generate pathological inflammatory responses to BV will result in having PROM and/or going into PTL (Denney and Culhane, 2009). Accordingly, the abnormal vaginal flora generally associated with BV would result in different lengths of gestation in susceptible women (Pretorius *et al*., 2007). Children may present with long-term neurological effects, such as cerebral palsy, hyperactivity, developmental delays, severe handicaps and preventricular leucomalacia (Grether and Nelson, 2000; Ling *et al*., 2004; Turovskiy *et al*., 2011).

2.4.9 Diagnosis of bacterial vaginosis

The gold standard for the detection of BV is based on clinical and laboratory diagnoses, the Amsel criteria and the Nugent scoring system, respectively (Danielsson *et al*., 2011). Various other tests enable the diagnosis of BV; however, these tests have either low sensitivity and/or specificity or are more expensive than the gold standard (Livengood, 2009). An example includes the Papanicolaou smear, which has been found to be a poor screening test with a sensitivity ranging from 50% to 89% and a specificity of around 90% (Greene *et al*., 2000; Sodhani *et al*., 2005).

2.4.9.1 Diagnosis by wet-mount microscopy

Wet-mount microscopy relies on phase-contrast microscopy and may be more rapid and accurate than the Gram-stain by identifying the characteristic granular flora pattern of *G. vaginalis/Bacteroides* spp. morphotypes (Donders, 2007). Donders *et al*. (2000) suggested that the staining process damages some of the lactobacillary flora and by this means favours the non-lactobacillary flora. The normal vaginal lactobacillary flora is better visualised with wet mounts than with the Gram-stain (Donders, 2007). However, wet-mount microscopy is subjective due to inter-observer variability and the preparation of the wet-mount (Schoeman, 2002). Therefore, it would be of more value in the case of skilled microscopists (Schoeman, 2002).

2.4.9.2 The Nugent scoring system

The laboratory diagnosis of a clinical condition with Gram-stained smears was first done by Spiegel *et al*. (1983) but was refined by Nugent *et al*. (1991) who established the Nugent scoring system (Donders, 2007). The Nugent scoring system is a system where Gram-stained slides are microscopically analysed, which is mainly based on the presence or absence of lactobacilli (Figure 2.4) (Brotman and Ravel, 2008; Denslow *et al*., 2011).

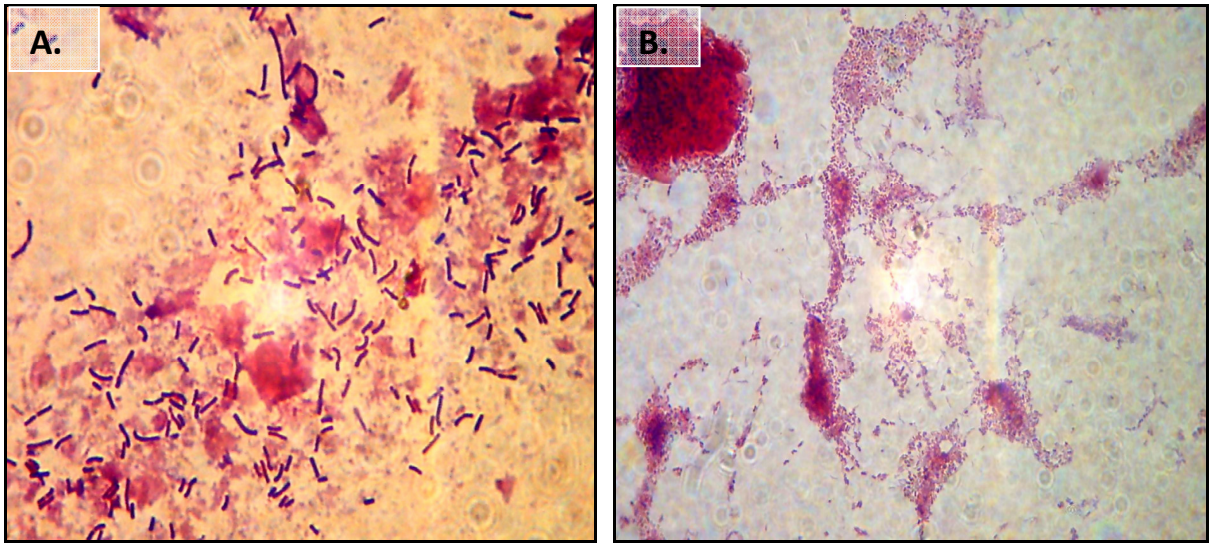

Figure 2.4: Microscope images of Gram-stained vaginal smears from (A) a healthy woman (Nugent score = 0) with a *Lactobacillus* dominated vaginal environment (100X objective) and (B) a BV-affected woman (Nugent score = 10) with *G. vaginalis/Bacteroides* spp. morphotypes dominating the vaginal environment, appearing as a granular flora pattern on the slide (10X objective)

The different cell types are counted (*Mobiluncus* spp., *G. vaginalis/Bacteroides* spp. and *Lactobacillus* spp.) and a score between zero and ten is obtained; whereby a score of seven to ten corresponds to BV (Figure 2.4B), a score of four to six is considered intermediate (partial BV) and a score of zero to three indicates an undisturbed vaginal microflora (Figure 2.4A) (Nugent *et al*., 1991). Intermediate scores may indicate the development of BV or a woman that is being cleared of this disease entity; however, these 'intermediate flora' remains contentious (Donders, 2007).

Cultivation-independent methods indicated that many healthy women lack high numbers of lactobacilli and the healthy environment is maintained by other bacteria (Zhou *et al*., 2004). This may give rise to misleading Nugent scores and incorrect diagnosis. Another drawback of the Nugent scoring system is that it lacks sensitivity when *A. vaginae* is investigated because this bacterium is not readily detected by Gram-staining due to its erratic morphology (Witkin *et al*., 2007; Brotman and Ravel, 2008).

2.4.9.3 Ison and Hay scoring system

Ison and Hay (2002) established another grading system for Gram-stained vaginal smears. Instead of grading and allocating a score to individual bacterial species, the Ison and Hay grading system assigns a grade to a mixed group of bacteria, depending on the numerical contribution of individual morphotypes (Ison and Hay, 2002). Where only *Lactobacillus* morphotypes (normal flora) are present, a smear will be graded as grade I. Grade II comprises intermediate flora, which include reduced *Lactobacillus* morphotypes with diverse bacterial morphotypes, whereas grade III (BV) contains mixed bacterial morphotypes with few or no *Lactobacillus* morphotypes (Ison and Hay, 2002). Grade 0 will be smears that contain epithelial cells with no bacteria and in which case antibacterial agents in the vagina might be present (Ison and Hay, 2002). Grade IV contains epithelial cells enclosed in Gram-positive cocci only (Ison and Hay, 2002).

The Ison and Hay criteria have been refined by Verhelst *et al*. (2005). Verhelst and colleagues (2005) subdivided the grade I category into grade Ia, grade Ib and grade Iab and proposed a new category called grade I-like (Verhelst *et al*., 2005). Specimens containing only *L. crispatus* cell types (short, plump, darker-stained rods) were categorised as grade Ia, those containing only other *Lactobacillus* cell types (smaller/elongated and less stained than

in grade Ia smears) were categorised as grade Ib and specimens containing both *L. crispatus* and other lactobacilli were categorised as grade Iab (Verhelst *et al*., 2005). The grade I-like category contains short Gram-positive rods that are unevenly shaped with curved ends and may appear as Chinese letters (diphtheroid cell types) (Verhelst *et al*., 2005).

2.4.9.4 Amsel's criteria

Amsel *et al*. (1983) described four criteria for the diagnosis of BV in clinical settings. A fulfilment of at least three of the four criteria for women is needed to be clinically diagnosed with BV (Amsel *et al*., 1983). Amsel's criteria include (i) vaginal pH >4.5, (ii) a thin homogeneous vaginal discharge, (iii) a fishy odour on the addition of 10% potassium hydroxide (KOH; whiff test) and (iv) clue cells present on wet-mount microscopy (Amsel *et al*., 1983; Turovskiy *et al*., 2011).

The shortcomings of Amsel's criteria are several. The whiff test may be subjective as a fishy odour is not always present, even after the application of KOH and interpretation may vary between investigators (Donders, 2007). A vaginal discharge has been reported to have low sensitivity (56%) and specificity (49%) and is present in only ±50% of BV positive women (Schwiertz *et al*., 2006; Donders, 2007). Additionally, a raised vaginal pH may be the result of several other lower genital tract conditions or due to vaginal and cervical secretions (Nelson and Macones, 2002). Clue cells may be difficult to be recognised as these cells can be entirely, partially, or not at all covered by anaerobic flora and *G. vaginalis* morphotypes (Marconi *et al*., 2012). Marconi *et al*. (2012) highlighted that a granular flora pattern (Figure 2.4B) is more indicative of BV than to search for clue cells.

2.4.9.5 Culture and PCR detection of bacteria associated with bacterial vaginosis

Commercial media are available for the cultivation of BV-associated bacteria, such as *Gardnerella* agar for *G. vaginalis* and Chocolate agar for anaerobes (*Bacteroides/Mobiluncus* spp.) (Goffinet *et al*., 2003). Cultures of bacteria, such as *G. vaginalis* are of no value for BV diagnosis as women who are merely colonised with this bacterium will also have positive cultures, whereas other bacteria, such as *A. vaginae* are fastidious, which makes cultivation difficult (Donders, 2007; Trama *et al*., 2008). Molecular detection methods may be more expensive than the gold standard but allow better characterisation of the vaginal flora by targeting genes (mainly the 16S rRNA sequence) of specific bacterial genera or species

(Fredricks *et al*., 2007). The molecular detection of fastidious bacterial species has been shown to be a more consistent indicator of BV than the detection of bacteria, such as *G. vaginalis*, which is present in patients without BV (Fredricks *et al*., 2007). *Atopobium vaginae, Leptotrichia* BVAB1-3, *Megasphaera* spp. and *Sneathia* spp. are examples of bacterial species that have been identified by means of molecular techniques (Fredricks *et al*., 2005; Verhelst *et al*., 2005). Multiplex quantitative PCR (qPCR) assays may aid in elucidating the pathogenic or protective roles bacteria play in health and disease (Mernard *et al*., 2010; Zozaya-Hinchliffe *et al*., 2010).

2.4.10 Treatment and prevention of bacterial vaginosis

It is anticipated that if BV increases women's susceptibility to HIV infection, interventions to reduce the occurrence of BV will have an impact on the prevention of the spread of HIV at a population level (Myer *et al*., 2005). Metronidazole and clindamycin (antimicrobial agents that are active against anaerobic bacteria) are the preferred treatment for BV as recommended by the CDC, with a cure rate of 80% to 90% within one week (Armstrong and Wilson, 2010; CDC, 2010). Even though the use of metronidazole in the first trimester was previously discouraged due to its potential for teratogenicity (causing developmental anomalies in the foetus), both antimicrobial agents are said to be safe to use in pregnancy (CDC, 2010; Sobel *et al*., 2012). In South Africa, symptomatic women, i.e. presenting with a vaginal discharge, are treated according to the VDS flowchart (Figure 2.5) (Lewis and Maruma, 2010).

According to the VDS flowchart, if a woman has not been sexually active for three months she is treated with 2 g oral metronidazole for BV in combination with clotrimazole for *Candida* infections (Lewis and Maruma, 2010). These women are at a very low risk of having STIs (Lewis and Maruma, 2010). If a pregnant woman is presenting with a vaginal discharge without any abdominal pain then treatment consists of: (i) an oral single 400 mg dose cefixime, (ii) 500 mg amoxicillin (orally), three times daily for 7 days and (iii) oral 2 g metronidazole (Lewis and Maruma, 2010). Pregnant women who are sensitive to penicillin may use a single dose of 2 g spectinomycin (an aminoglycoside) instead of cefixime and 500 mg oral doses of erythromycin, six hourly for seven days, instead of amoxicillin (Lewis and Maruma, 2010).

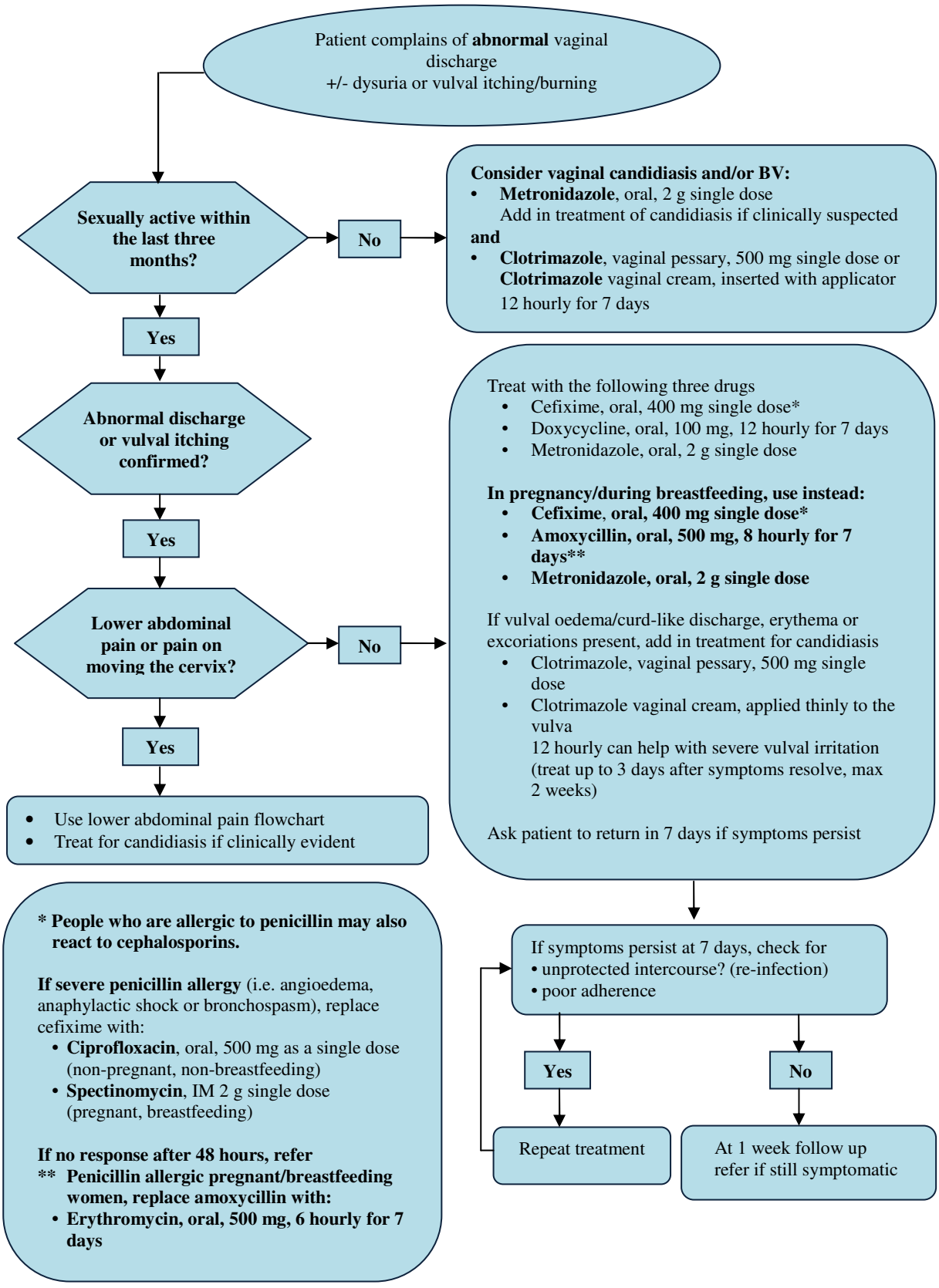

Figure 2.5: Vaginal Discharge Syndrome flowchart as adapted from the Primary Health Care Standard Treatment Guidelines and Essential Drugs List of South Africa (Lewis and Maruma, 2010)

Ferris and colleagues (2004) demonstrated that *A. vaginae* has resistance to metronidazole but is susceptible to clindamycin. With *A. vaginae* being one of the major pathogenic contributors of BV, it is expected that clindamycin would be more effective in the treatment of BV positive women (Bradshaw *et al*., 2006). However, it has been shown that metronidazole and clindamycin have equal short-term effectiveness in the treatment of BV (Koumans *et al*., 2007). A possible reason is that following treatment with metronidazole, the decline in metronidazole-sensitive species can lead to a concurrent decline in metronidazole-resistant species (Bradshaw *et al*., 2006). Retrocyclin 101 (RC-101), a cyclic antimicrobial peptide, has been shown to strongly inhibit the cytolytic activity of vaginolysin and biofilm formation of *G. vaginalis in vitro* and is a potential candidate for the treatment and prevention of BV (Hooven *et al*., 2012).

The undesirable pregnancy outcomes and costs associated with affected neonates call for more effective drugs and preventative methods as treatment fails in up to 50% of cases (Schoeman, 2002; Hay, 2010). McDonald *et al*. (2007) published a review of 15 trials and determined that the administration of antimicrobial agents may have eliminated BV in pregnancy but did not decrease the risk of preterm birth.

Bacterial vaginosis can be prevented by limiting the number of sexual partners and avoiding douching and thereby not disrupting the normal vaginal flora (CDC, 2013). The occurrence of BV may be reduced by the use of condoms (Fethers *et al*., 2008) and the completion of a course of antibiotics may prevent relapse (CDC, 2013).

2.5 Genital mycoplasmas

Mycoplasmas are small self-replicating bacteria that lack cell walls and often inhabit the genital tracts of men and women (Goering *et al*., 2008; Waites *et al*., 2009). It is suggested that sexually experienced women are more prone to colonisation than men (Agbakoba *et al*., 2011). Genital mycoplasmas play important roles in foetal, neonatal and maternal health as these bacteria are able to enter and infect the placental and foetal tissues (Waites *et al*., 2009; Larsen *et al*., 2010).

2.5.1 History of genital mycoplasmas

The first report of a mycoplasma to be recovered directly from a human and associated with a pathological condition was in 1937 by Dienes and Edsall (Hartmann, 2009). Several other human mycoplasmal species were described since and in 1954 Shepard and colleagues provided the first description of Tiny (T)-strain mycoplasmas, later known as ureaplasmas (Yoshida *et al*., 2007). In the 1960s it was proven that mycoplasmas were unable to produce cell walls under any circumstance, distinguishing these bacteria from bacterial L (Lister) forms (Razin and Hayflick, 2010). In the 1960s, the mycoplasmas and associated bacteria were collectively grouped into the class *Mollicutes* (Waites *et al*., 2005).

The debates in the 1960s concerning the structure, cell size and mode of mycoplasma replication have dwindled in the 1970s as most aspects have been agreed on (Razin and Hayflick, 2010). In 1981, *M. genitalium* was isolated from men with nongonococcal urethritis (NGU) by growing it in culture medium (Hartmann, 2009). However, because this bacterium is fastidious, it was only with the evolvement of molecular methods that its detection rate increased and its causative role in non-chlamydial, nongonococcal urethritis (NCNGU) became clear (Hartmann, 2009). *Ureaplasma urealyticum* consisted of two biotypes, biovar 1 and biovar 2, which were designated as separate species in 1999 namely *U. parvum* and *U. urealyticum*, respectively (Yoshida *et al*., 2007).

2.5.2 Classification of genital mycoplasmas

Genital mycoplasmas are contained within the phylum *Tenericutes* and class *Mollicutes* (Table 2.1), which are comprised of four orders, five families, eight genera and nearly 200 identified species that have been detected in humans, insects, animals and plants (Waites *et al*., 2012). Phylogenetic studies have shown that mycoplasmas have developed from clostridial-like Gram-positive cells by means of degenerative evolution (Hartmann, 2009). The genus *Mycoplasma* includes three species commonly found in the female genital tract, namely *M. fermentans, M. genitalium* and *M. hominis* (Waites *et al*., 2009). The two *Ureaplasma* species, with DNA sequence homology of less than 60%, contains a total number of fourteen serovars (Cao *et al*., 2007). Serovars 1, 3, 6 and 14 belong to the species, *U. parvum,* whereas serovars 2, 4, 5, 7 to 13 belong to the species, *U. urealyticum* (Xiao *et al*., 2010).

Table 2.1: The scientific classification of genital mycoplasmas (Edward, 1955; Tully *et al*., 1983; Robertson *et al*., 2002)

RANK	NAME	
Domain	*Bacteria*	
Phylum	*Tenericutes*	
Class	*Mollicutes*	
Order	*Mycoplasmatales*	
Family	*Mycoplasmataceae*	
Genus	*Mycoplasma*	*Ureaplasma*
Species	*hominis* and *genitalium*	*urealyticum* and *parvum*

In a study done by Moser *et al*. in 2006, pulsed field gel electrophoresis (PFGE) was used to discriminate between the different serovars of *Ureaplasma* to assess genetic relatedness (Figure 2.6). *Ureaplasma urealyticum* serovars 7 and 11 were identical at 100% similarity, whereas serovars 4 and 12 were 91% similar (Moser *et al*., 2006).

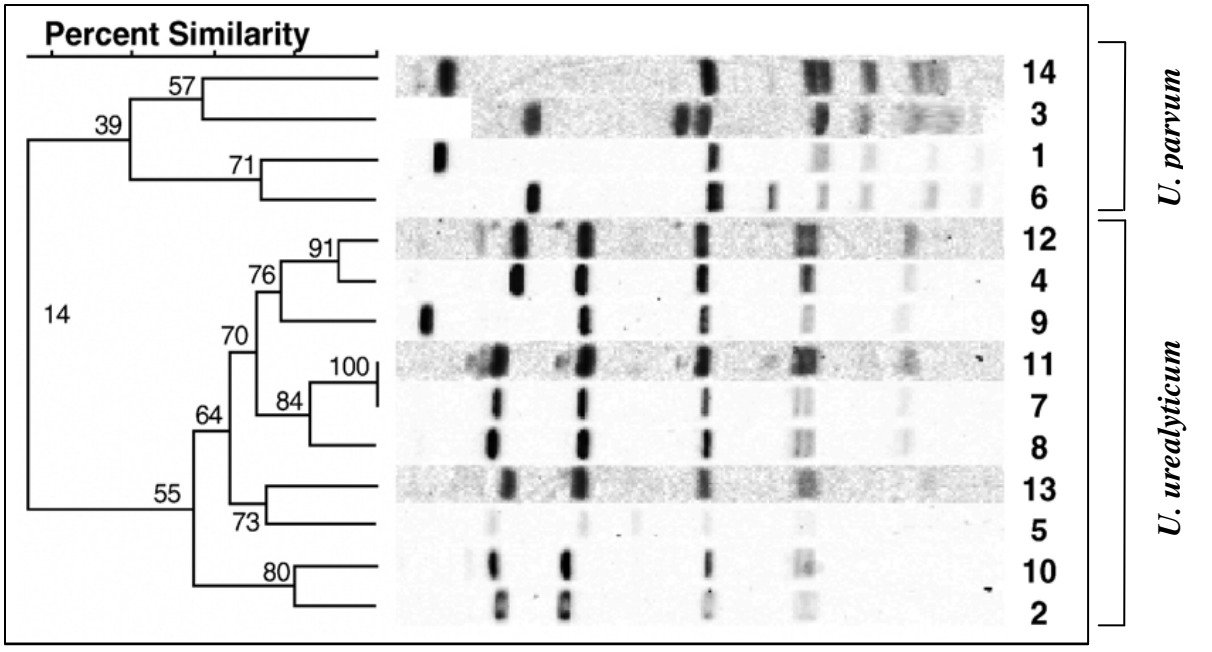

Figure 2.6: Dendrogramme constructed from PFGE banding patterns indicating the genetic relatedness of the 14 serovars of *Ureaplasma* spp. (Moser *et al*., 2006)

Serovars 2, 5, 8, 9, 10 and 13 were divergent to these groups and to each other (Moser *et al*., 2006). *Ureaplasma parvum* serovars 1 and 6 were 71% similar, while serovars 3 and 14 were more related but divergent to the latter group (Moser *et al*., 2006).

2.5.3 Characteristics of genital mycoplasmas

Mycoplasmas are facultative anaerobic bacteria that are typically spherical or coccobacillary-shaped cells (Ward *et al*., 2009). Mycoplasmas exist both intra- and ectocyticly in association with eukaryotic cells, establishing themselves on the mucosal surfaces of predominantly the urogenital and respiratory tracts (Waites *et al*., 2009). The lack of a rigid cell wall makes mycoplasmas susceptible to hostile environmental conditions, such as osmotic shock, dehydration, major temperature variations and toxic metabolites (Hartmann, 2009).

The genomes of mycoplasmas resemble that of a typical prokaryote but are very small in size, limiting their spectrum of metabolic capabilities (Razin and Hayflick, 2010). It consists of three fundamental organelles: a cell membrane, ribosomes and a circular, compact, double-stranded DNA molecule, which allows mycoplasmas to self-reproduce (Razin and Hayflick, 2010). Mycoplasmas hydrolyse different substrates to generate metabolic energy for self-utilisation (Stein and Baseman, 2005). *Mycoplasma hominis* metabolises arginine, while *Ureaplasma* spp. and *M. genitalium* metabolise urea and glucose, respectively (Stein and Baseman, 2005).

The genomes of mycoplasmas are approximately 500 to 1 200 kilobase pairs (kb) (Zeighami *et al*., 2007). The genome of *U. urealyticum* serovar 3 was the third mycoplasmal genome to be sequenced and comprises 751 719 base pairs (bp) and contains 639 genes (Juhász *et al*., 2011). *Ureaplasma parvum* contains all the small genome serovars, ranging in size from 0.75 to 0.76 megabase pairs (Mb), whereas *U. urealyticum* comprises the large genome serovars ranging in size from 0.88 to 1.2 Mb (Juhász *et al*., 2011). The genome of *M. genitalium* is the smallest (580 kb) with only 382 of 482 protein-coding genes identified as essential, indicating how little DNA is required to sustain microbial life (Glass *et al*., 2006; Larsen and Hwang, 2010). Mycoplasmas have a low G+C content in its DNA, which ranges from 24% to 33% (Jensen, 2006).

2.5.4 Epidemiology of genital mycoplasmas

Genital mycoplasmas are primarily transmitted *via* sexual contact, i.e. venereally (Agbakoba *et al*., 2011) but transmission may be vertical from mother to offspring, either *in utero* or during the passage through the birth canal (Pandey *et al*., 2007; Taylor-Robinson, 2007). Infection *in utero* is more likely to occur after the rupture of amniotic membranes than with intact membranes (Taylor-Robinson and Lamont, 2011). Vertical transmission of *M. genitalium* from mother to foetus is rarely reported (Taylor-Robinson and Lamont, 2011). Colonisation with genital mycoplasmas is higher after puberty and sexual maturity and is increased in relation to the number of sexual partners (Taylor-Robinson and Lamont, 2011). Other predisposing factors for colonisation with genital mycoplasmas include low socioeconomic status, smoking, younger age, African-American ethnicity and oral contraceptive use (Zeighami *et al*., 2007).

Ureaplasma spp. are more prevalent than other mycoplasmas in the female urogenital tract with a prevalence of up to 80% in healthy sexually active adults (Juhász *et al*., 2011). *Ureaplasma parvum* is isolated about four times more often than *U. urealyticum* (Patel and Nyirjesy, 2010). Both species may occur simultaneously and in more than a third of the cases, more than one serotype can be detected (Waites *et al*., 2005). Ureaplasmas colonise roughly one third of infant girls and are present in up to 22% of prepubertal girls (Taylor-Robinson, 2007). *Mycoplasma hominis* is less prevalent in these populations and a prevalence of 17% in prepubertal girls has been reported (Taylor-Robinson, 2007). This species is habitually found in the cervix or vagina of sexually mature asymptomatic women with colonisation rates as high as 53% (Waites *et al*., 2005). *Mycoplasma genitalium* is reported to be prevalent in around 3% of sexually active young adults (Andersen *et al*., 2007; Hamasuna *et al*., 2008). Hitti *et al*. (2010) found this species to be significantly ($p=0.003$) associated with younger maternal age.

Several researchers investigating the prevalence of mycoplasmas in sexually active women reported similar results (Schlicht *et al*., 2004; Amirmozafari *et al*., 2009; Larsen and Hwang, 2010). Schlicht and colleagues (2004) found a prevalence of *Ureaplasma* spp., *M. hominis* and *M. genitalium* in 65 sexually active young adults to be respectively 37%, 13% and 8%, with urethritis or cervicitis symptoms. In an Iranian study comprising 210 women with any genital infection, the prevalence of *Ureaplasma* spp. were found to be 32.3% and that of

Mycoplasma spp. 13.3% (Amirmozafari *et al*., 2009). Another study in 2006 to 2007 by McIver and co-workers (2009) reported a prevalence of *Ureaplasma* spp., *M. hominis* and *M. genitalium* in 175 sexually active Australian women to be 64%, 13.7% and 1.3%, respectively. A study in London including more than 1 200 pregnant women reported a colonisation rate of only 0.7% by *M. genitalium* (Larsen and Hwang, 2010). Ureaplasmas can be isolated from 82% of women during pregnancy and in 24% of women in the puerperium (Juhász *et al*., 2011).

2.5.5 Virulence factors of genital mycoplasmas identified

The most essential virulence factors of genital mycoplasmas are possibly those that initiate inflammatory responses (Larsen and Hwang, 2010). However, these opportunistic pathogens need extra factors to achieve their virulent potential (Larsen and Hwang, 2010). The proposed virulence factors for *Ureaplasma* spp. include urease, immunoglobulin-α (IgA) protease, adhesins, phospholipases A1, A2 and C, the Multiple-banded antigen (MBA) and the *Ureaplasma* enzymatic machinery for the generation of hydrogen peroxide (Viscardi and Hasday, 2009; Juhász *et al*., 2011). *Ureaplasma parvum* has genes for two haemolysins, *hly*A and *hly*C, encoding haemolysins A and C, respectively (Juhász *et al*., 2011). Haemolysin A has been suggested as a new virulence factor for *Ureaplasma* spp. and orthologues of this haemolysin in other microorganisms have both haemolytic and cytotoxic activity (Waites *et al*., 2005; Juhász *et al*., 2011).

Virulence factors of *M. hominis* include adhesins P100 and the variable adherence-associated (Vaa) antigen, an adhesin that varies in size and phase and is highly immunogenic (Hartmann, 2009; Waites *et al*., 2009). The virulence factors of *M. genitalium* include its terminal tip organelle with adhesins [the most widely characterised is the 140 kDa *M. genitalium* adhesin protein (MgPa)], enzymes and the ability to escape the immune response produced by the host by antigenic variation (Razin *et al.*, 1998; Ueno *et al*., 2008). Two enzymes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and methionine sulfoxide reductase (MsrA), play important roles in the pathogenesis of *M. genitalium* infections (Dhandayuthapani *et al*., 2001; Alvarez *et al*., 2003).

Adhesin proteins of *M. hominis* and *Ureaplasma* spp. have not yet been characterised and structured into a perceptible organelle as opposed to those of *M. genitalium* (Waites *et al*.,

2009). The ability of *Ureaplasma* spp. and *M. hominis* to metabolise urea and arginine, respectively, have been proposed as potential virulence factors (Waites *et al*., 2005).

2.5.6 Pathogenesis of genital mycoplasmas

The mechanisms and processes involved in the pathogenesis of genital mycoplasmas are still poorly understood. Damage related to mycoplasma infections may be the result of the induced host immune and inflammatory responses rather than the direct toxic effects of mycoplasma cellular components (Razin and Hayflick, 2010).

Adhesion of mycoplasmas to the host epithelial cells, facilitated by membrane adhesin proteins is vital for colonisation and successive infection (Hartmann, 2009; Taylor-Robinson and Lamont, 2011). Due to the variation of these surface protein antigens, mycoplasmas evade immune responses and may persist in invasive sites (Taylor-Robinson and Lamont, 2011). Ureaplasmas can use IgA protease to gain access to the upper urogenital tract by degrading IgA, the primary immune component of mucosal surfaces (Waites *et al*., 2009). By binding to neutrophils, these bacteria can activate the first component of the complement system (Juhász *et al*., 2011). Apart from the terminal tip organelle, the attachment of *M. genitalium* to vaginal and cervical surfaces is assisted by the enzyme GAPDH (Alvarez *et al*., 2003).

The surrounding fragile cell membrane of mycoplasmas makes these bacteria extremely sensitive to growth inhibition and lysis by the complement system and antibodies (Razin and Hayflick, 2010). Despite this, mycoplasmas often evade destruction by the host defence mechanisms through variable switching of the expression and alteration of key membrane protein antigens (Razin and Hayflick, 2010). The great variability of the Vaa antigen may contribute to the diversity and host adaptation of *M. hominis* (Waites *et al*., 2009). Similar to the MBA of *Ureaplasma* spp., the Vaa may be involved in the stimulation and evasion of the host inflammatory response (Juhász *et al*., 2011). *Mycoplasma genitalium* uses its MsrA enzyme to resist host oxidative damage by restoring proteins that have lost their activity due to such damage (Dhandayuthapani *et al*., 2001).

Through the hydrolysis of urea and arginine, secretory products, such as ammonia (NH_3) are released into the vaginal milieu (Waites *et al*., 2005). The production of large quantities of

NH_3 by both the urease activity and arginine depletion may generate local cytotoxic effects (Waites *et al*., 2005). García-Castillo *et al.* (2008) reported biofilm formation in *Ureaplasma* spp., which can protect mycoplasma cells from host defences and antimicrobial agents.

The hypothesised sequence of events leading to adverse pregnancy outcomes implicates bacterial toxins and the mycoplasma membrane lipoproteins (Pararas *et al*., 2006). These components stimulate the production of cytokines by the foetal membranes and decidua (the mucous membrane lining the uterus), which in turn activates the production and subsequent release of prostaglandins (Challis *et al*., 2009). The eventual production of proteases and other substances is responsible for pregnancy complications (Taylor-Robinson and Lamont, 2011). Ureaplasmal phospholipases contribute to PTL by altering the biosynthesis of prostaglandin and arachidonic acid (Glass *et al*., 2000).

2.5.7 Clinical manifestations due to genital mycoplasma infections

Genital mycoplasmas can cause cervicitis and PID, are associated with women with cervical insufficiency and can lead to infertility in affected women (Lee *et al*., 2008; Xiao *et al*., 2010). These bacteria are frequently isolated from the amniotic fluid of pregnant women and are linked to poor pregnancy outcomes with short- and long-term effects (Kacerovsky *et al*., 2011). Similar to BV, poor pregnancy outcomes are associated with infection of the cervix, amniotic membranes, amniotic fluid, placenta or umbilical cord (Larsen and Hwang, 2010). Genital mycoplasmas ascend from the lower genital tract to penetrate the chorioamnion, cause chorioamnionitis and invade the amniotic cavity to infect the foetus (Taylor-Robinson and Lamont, 2011).

Mycoplasma hominis is specifically associated with conditions, such as endometritis, PROM, PTB and spontaneous abortions (Witt *et al*., 2005; Kataoka *et al*., 2006). Information on *M. genitalium* in pregnancy is limited but this species has been associated with endometritis, post-abortal salpingitis and spontaneous preterm birth (Haggerty *et al*., 2006; Hitti *et al*., 2010). In pregnancy, *Ureaplasma* spp. infection can lead to chorioamnionitis, PROM, spontaneous abortion, PTB, stillbirth and postpartum endometritis (Stellrecht *et al*., 2004; Schelonka and Waites, 2007; Larsen and Hwang, 2010). *Ureaplasma urealyticum* is isolated

from pathogenic cases more often than *U. parvum* (Abele-Horn *et al*., 1997; Zdrodowska-Stefanow *et al*., 2006).

In neonates, mycoplasma infections are associated with chronic lung conditions, such as bronchopulmonary dysplasia (BPD), pneumonia and respiratory distress syndrome (Waites *et al*., 2005; Goldenberg *et al*., 2008). In addition, genital mycoplasmas have been linked to LBW, meningitis and intraventricular haemorrhage (Waites *et al*., 2005; Olomu *et al*., 2011).

2.5.8 Laboratory diagnosis of genital mycoplasmas

The gold standard method for the detection of genital mycoplasmas from clinical specimens is culture (Zeighami *et al*., 2007). However, the low sensitivity of conventional culture methods and the fastidious growth of species, such as *M. genitalium* have led to the development and routine use of PCR-based identification methods (Waites *et al*., 2009).

2.5.8.1 Culture methods used to detect genital mycoplasmas

Clinical specimens suitable for the culture of genital mycoplasmas include tissues, swabs and body fluids, such as urine (Waites *et al*., 2009). Mycoplasmas are cell-associated and therefore, when swabs are used for specimen collection, the preferred site of collection must be swabbed vigorously to obtain as many cells as possible (Waites *et al*., 2009). After collection, specimens should be inoculated into a suitable transport and/or culture medium as soon as possible to prevent drying (Waites *et al*., 2009). Media used for transport and/or growth may include 10B/U9 broth, Stuart's medium or Mycotrans (Waites *et al*., 2005; Waites *et al*., 2009).

Ureaplasma spp. use the urease enzyme to metabolise urea incorporated into the U9 broth (Hartmann, 2009). Arginine broth is used to select for *M. hominis* and contains arginine (Hartmann, 2009). These liquid media contain phenol red, which acts as a colour indicator for growth (Juhász *et al*., 2011). Colour changes may be visible between 48 h and one week after which a corresponding solid medium is used to confirm growth (Waites *et al*., 2005). The limited biosynthetic capabilities of mycoplasmas call for complex growth media, such as A2, A7 or A8 agar media (Shepard and Lunceford, 1976; Waites *et al*., 2005). These media commonly combine a rich nutritive base containing yeast extract, peptones, horse serum and growth factors (cysteine, arginine, urea) favouring the growth of mycoplasmas (Fiacco *et al*.,

1984; Razin and Hayflick, 2010). The difficulty to cultivate bacteria like *M. genitalium* is due to a lack of genes responsible for amino acid synthesis (Himmelreich *et al*., 1997). The overgrowth of other microorganisms is prevented by including antimicrobial agents, such as penicillin, amphotericin, cefoperazone or nystatin in the growth media (Broitman *et al*., 1992; Krausse and Schubert, 2010).

Agar plates should be incubated in an atmosphere of 5% to 10% CO_2 at 37ºC for two to five days (Gdoura *et al*., 2007). The surface of the agar is infiltrated by the mycoplasmas to gain access to the underlying agar where these bacteria grow (Hartmann, 2009). Colonies of *M. hominis* have a "fried-egg" appearance and are 200 µm to 400 µm in size, whereas *Ureaplasma* colonies are brown granular colonies and are 15 µm to 60 µm in size (Figure 2.7) (Waites *et al*., 2005).

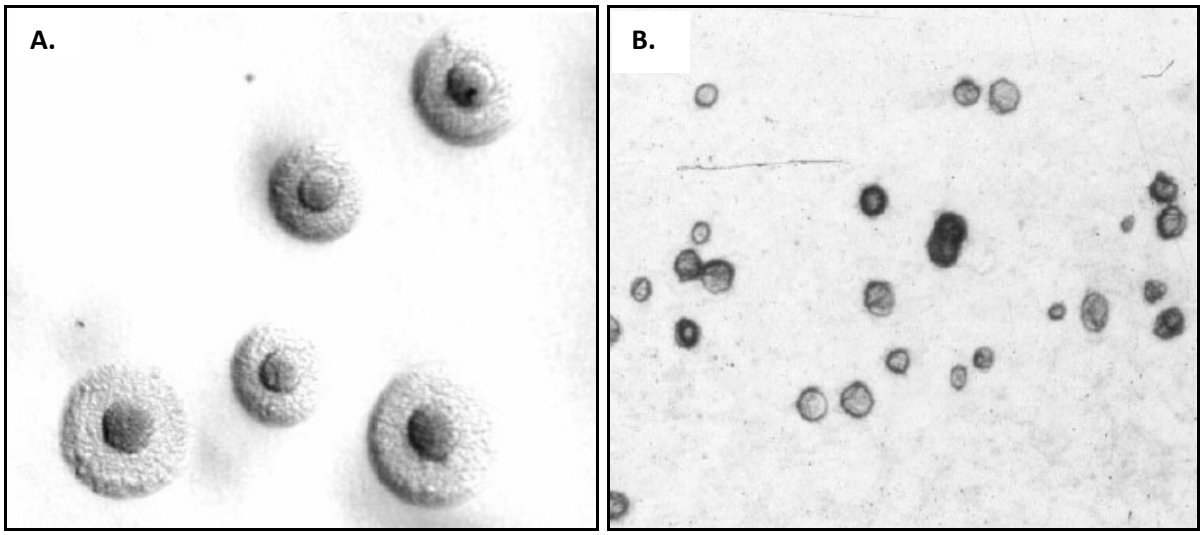

Figure 2.7: Stereomicroscope images (126X objective) of mycoplasma growth on A8 agar medium. (A) Characteristic "fried-egg" colonies of *M. hominis* and (B) Subsurface granular colonies of *Ureaplasma* spp. (Waites *et al*., 2005)

Culture methods are laborious and less sensitive when compared to molecular methods, such as PCR assays (Volgmann *et al*., 2005). Contamination of cultures with other microorganisms is very common and culture requires constant observation with light microscopy (Petrikkos *et al*., 2007). Mycoplasmas are fastidious bacteria and may in total take up to 12 days to be diagnosed (Waites *et al*., 2012). Culture can only give 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). Nevertheless, culture media can be used one at a time and do not need to be batched, whereas PCR is more cost-effective if run in batches (Waites *et al*., 2012). In laboratories with low to moderate test volumes, batching will prolong the turnaround time for result output and reporting (Waites *et al*., 2012). An additional value of culture is that isolates can be used to perform antimicrobial susceptibility testing (Waites *et al*., 2012).

2.5.8.2 Molecular methods used for detecting genital mycoplasmas

Polymerase chain reaction assays (conventional and real-time) allow for the rapid detection of infectious agents and do not require the use of viable organisms (Gdoura *et al*., 2008). These methods are not only more rapid than culture but can be more sensitive and specific with the use of less-invasive clinical specimens, such as first-void urine (FVU) (Sung *et al*., 2006; Cao *et al*., 2007).

The 16S rRNA, 16S-23S rRNA intergenic spacer region, genus-defining urease and serovar-defining MBA genes of ureaplasmas are common targets in PCR-based assays (Mallard *et al.*, 2005; Haggerty *et al*., 2009; Woodman *et al.*, 2011). The 16S rRNA and a house-keeping gene, glyceraldehyde-3-phosphate (*gap*), of *M. hominis* have been used as gene targets (Waites *et al*., 2005; Sung *et al*., 2006; McIver *et al*., 2009). Targets for *M. genitalium* include the *gap*, 16S rRNA and MgPa adhesin genes or short tandem repeats (STRs) (Pollack *et al*., 2002; Taylor, 2005). However, the entire genetic sequence of the MgPa adhesin gene may be altered when the gene undergoes antigenic variation, which restricts the use of this gene as a PCR target (Ueno *et al*., 2008).

Real-time PCR assays have been developed to differentiate between the two *Ureaplasma* spp. (Yi *et al*., 2005; Cao *et al*., 2007). Compared to culture systems and conventional PCR assays, real-time PCR assays have reduced carry-over contamination, are more rapid and have a higher sensitivity and specificity (Mallard *et al*., 2005; Xiao *et al*., 2010). The detection chemistries typically used in mycoplasma real-time PCR assays include TaqMan probes, molecular beacons, SYBR green and hybridisation probes (Yoshida *et al*., 2007; Waites *et al*., 2012). The binding of a third oligonucleotide probe to target sequences increases the specificity of real-time PCR assays (Waites *et al*., 2012). These assays offer the opportunity of quantification with the determination of bacterial load (Xiao *et al*., 2010). Bacterial load

can indicate whether the bacterium is a coloniser or infectious agent according to pathological thresholds (Waites *et al*., 2012).

2.5.8.3 Commonly used commercial diagnostic assays and antimicrobial susceptibility testing of genital mycoplasmas

Commercial diagnostic assays are available for the detection and identification of genital mycoplasmas and simultaneous antimicrobial susceptibility testing (Tarrant *et al*., 2009). Commonly used commercial assays include the Mycoplasma Duo Kit (Sanofi Diagnostics Pasteur, France), Mycofast US (ELiTech Diagnostic), the Mycoview (Ivagen), the MycoIST2 (BioMérieux), the Mycofast Evolution 3 (ELiTech Diagnostic) and the Mycofast Revolution (ELiTech Diagnostic) assays (Cheah *et al*., 2005; Govender *et al*., 2009; Tarrant *et al*., 2009). The Mycofast Revolution assay is the latest of these assays and is the only assay, which allows antimicrobial susceptibility testing with specific minimum inhibitory concentrations (MICs) as defined by the 2011 Clinical and Laboratory Standards Institute (CLSI) guidelines. The antimicrobial agents that are tested include levofloxacin, moxifloxacin, erythromycin, clindamycin and tetracycline (CLSI, 2011).

The ease of performance and rapid detection (results within 24 h to 48 h) of these assays are attractive for routine diagnostic laboratories and researchers, as this allow for higher throughput of results (Amirmozafari *et al*., 2009). Most of these assays are based on liquid broth cultures and consist of an inoculation tray with wells that are pre-coated with growth media and antimicrobial agents (CLSI, 2011). Drawbacks of commercial detection assays include the restriction to phenotypic identification and only viable bacteria are detected.

2.5.8.4 Other tests used to diagnose genital mycoplasma infections

A number of tests detecting the antibodies of genital mycoplasmas exist. These include haemagglutination, immunofluorescence of colonies on agar, immunoperoxidase and enzyme-linked immunosorbent assays (ELISA) (Echahidi *et al*., 2000; Hartman, 2009; Xiao *et al*., 2010). These immunological methods are cumbersome and are often limited by the presence of intra-species cross-reactivity and sensitivity (Sung *et al*., 2006; Xiao *et al*., 2010).

2.5.9 Treatment and prevention of genital mycoplasma infections

Due to the lack of a cell wall, vancomycin and beta-lactam antimicrobial agents, such as penicillins and cephalosporins are ineffective for the treatment of mycoplasma infections (Waites *et al*., 2005; Hartmann, 2009). *Mycoplasma hominis* is naturally resistant to macrolides, such as clarithromycin and erythromycin, while *Ureaplasma* spp. have inherent resistance to clindamycin (Bébéar and Bébéar, 2002; Kechagia *et al*., 2008). Genital mycoplasmas are generally treated with macrolides, fluoroquinolones and tetracyclines, specifically doxycycline (Patel and Nyirjesy, 2010). First-line antimicrobial agents like doxycycline are contraindicated in pregnancy (Raynes-Greenow *et al*., 2011). Fluoroquinolones can be used when strains are resistant to doxycycline (Duffy *et al*., 2000). Alternatively, the macrolides, lincosamides, streptogramins, ketolides and oxazolidinones (MLSKO) group of antimicrobial agents are used (Roberts, 2008).

Erythromycin, a macrolide, is often empirically used during pregnancy (Koh *et al*., 2009). The administration of clindamycin (active against *M. hominis*) and macrolides (active against ureaplasmas) during the second trimester of pregnancy has been shown to reduce the rates of PTB (Morency and Bujold, 2007). The macrolide, azithromycin, is active against *M. genitalium*; ureaplasmas are less susceptible, while *M. hominis* is resistant (Dongya *et al*., 2008). Treatment failure for *M. genitalium* with azithromycin has been reported (Hartmann, 2009). In such cases women should be treated with moxifloxacin instead (Manhart *et al*., 2011). When compared to erythromycin, the pharmacokinetic properties of azithromycin permit the use of higher intracellular and tissue concentrations, fewer adverse effects and better tolerance (Juhász *et al*., 2011). Azithromycin is a more attractive antimicrobial agent although it has not been studied extensively in all populations (Juhász *et al*., 2011). Furthermore, erythromycin does not efficiently penetrate the amniotic sac (Waites *et al*., 2005). Clarithromycin, active against ureaplasmas and *M. genitalium* but not *M. hominis,* has a higher transplacental passage than other macrolides like roxithromycin, azithromycin and erythromycin (Heikkinen *et al*., 2000; Witt *et al*., 2003).

The mere presence of genital mycoplasmas is not enough reason to commence treatment (Taylor-Robinson, 2007). This might be different for *M. genitalium*, which has a higher pathogenic potential (Taylor-Robinson and Lamont, 2011). Due to the frequent association of genital mycoplasmas and BV, a syndromic approach with broad-spectrum antimicrobial

agents to cover the bacteria found in BV is recommended (Taylor-Robinson, 2007; Lewis and Maruma, 2010). Potential agents that may reduce the incidence of BV associated bacteria, including genital mycoplasmas, are clindamycin and clarithromycin (Taylor-Robinson, 2007; Lamont *et al*., 2011). Austin *et al*. (2005) reported that treatment with two antimicrobial agents, metronidazole and clindamycin, was effective at decreasing colonisation of *M. hominis* in women who were treated for BV.

The options for treatment of respiratory and systemic infections in neonates are limited. Macrolides are the most widely used for neonatal mycoplasmal and ureaplasmal infections (Waites *et al*., 2005). Azithromycin and clarithromycin are more often used than erythromycin due to better tolerability and the rare cases of long-term effects in children associated with erythromycin treatment (Waites *et al*., 2005). Reported long-term effects of the use of erythromycin in pregnancy include cases of cerebral palsy and cardiovascular toxicity (Kenyon *et al*., 2008; Dando *et al*., 2010). Tetracyclines are used to treat invasive infections of the cerebrospinal fluid (CSF) by *Ureaplasma* spp. and *Mycoplasma* spp. (Waites *et al*., 2005).

2.6 Summary

The association between genital mycoplasmas and BV is controversial. Many studies supporting an association between these bacteria failed to define the contributing role of these bacteria in the aetiology of BV (Bayraktar *et al*., 2010; Donati *et al*., 2010).

Hartmann (2009) reported that cultures from the vaginal smears of BV positive women indicated high titres of *M. hominis* and ureaplasmas, while it is reported that *M. genitalium* does not play any role in BV (Hitti *et al*., 2010). *Mycoplasma hominis* and ureaplasmas may possibly play a vital role in the pathogenesis of BV due to an independent association with this condition but these bacteria are not the causative species (Cedillo-Ramirez *et al*., 2000; Waites *et al*., 2005). Although ureaplasmas are associated with BV, these bacteria are less frequently implicated in BV than *M. hominis* (Taylor-Robinson, 2007). *Mycoplasma hominis* can be 10 000-fold more in numbers in women with BV than those without BV and larger numbers of this species are isolated in women with higher Nugent scores (7 to 10) (Taylor-Robinson and Lamont, 2011). De Francesco *et al*. (2009) investigated the relationship between the two *Ureaplasma* spp., *U. urealyticum* and *U. parvum* and vaginal flora changes.

Ureaplasma urealyticum and *U. parvum* serovars 3 or 14 were found more often in the absence of lactobacilli, while *U. parvum* serovar 6 was more associated with a normal vaginal flora (presence of lactobacilli). Keane *et al*. (2000) studied 38 women and found that only *M. hominis* was associated with BV positive women and not *Ureaplasma* spp. or *M. genitalium*. However, another study found no link between *M. hominis* and BV (Arya *et al*., 2001).

Ferris and colleagues (2007) reported high concentrations of *A. vaginae* in individuals that failed or responded incompletely to metronidazole treatment and the lowest in individuals that were cured. An accurate and reliable molecular tool for BV diagnosis was proposed by Menard and colleagues (2010) based on the combination of high vaginal quantification of *A. vaginae* and *G. vaginalis*. The real-time qPCR assay was sensitive (100%) and specific (93%) compared to the Nugent score as the reference method (Menard *et al.*, 2010). Even though the link between genital mycoplasmas and BV is unclear, it is reported that simultaneous infection of *M. hominis* and *U. urealyticum* with BV may cause more serious pregnancy outcomes, such as PTB or pregnancy loss (Taylor-Robinson and Lamont, 2011).

Information regarding the association between genital mycoplasmas and BV in pregnant women in South Africa, especially in the Pretoria region, is limited. Investigating the association between these bacteria may aid in defining the aetiological and pathogenic roles the various genital mycoplasma species play in BV. This data, together with the antimicrobial susceptibility profiles of genital mycoplasmas may indicate which therapeutic options would be the best to follow. Detection and early intervention of these RTIs/STIs are essential to prevent complications and adverse outcomes in pregnancies in an HIV-prevalent country, such as South Africa. This might minimise the risk for complications at a later stage, which may require the patient to seek further medical attention and lead to a greater financial burden. It may also reduce the rates of neonatal morbidity and mortality.

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CHAPTER 3

Comparison of the new Mycofast Revolution assay with a molecular assay for the detection of genital mycoplasmas from clinical specimens

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

Abstract

Background Genital mycoplasmas are opportunistic bacteria that are associated with undesirable gynaecologic and reproductive events. Mycoplasmas are fastidious bacteria with increasing resistance to routine antimicrobials and often fail to grow on conventional culture methods. The commercial Mycofast Revolution assay permits the phenotypic detection and identification of genital mycoplasmas. Antimicrobial susceptibility testing against five antimicrobial agents with MICs corresponding to the CLSI guidelines can also be performed. This study aimed to compare the new commercially available Mycofast Revolution assay with a multiplex PCR assay. **Methods** Self-collected swabs were obtained from pregnant women attending the antenatal clinic of a tertiary academic hospital in Pretoria, South Africa from October 2012 to November 2012. These swabs were used to seed UMMt and modified Amies transport media. The seeded UMMt transported medium was used to inoculate the Mycofast Revolution assay for the identification, enumeration and antimicrobial susceptibility testing of genital mycoplasmas. Following DNA extraction from the modified Amies transport medium, specimens were subjected to a multiplex PCR assay for the detection of genital mycoplasmas. **Results** The Mycofast Revolution kit had a sensitivity and specificity of 77.3% (95% CI: 62.15% to 88.51%) and 80% (95% CI: 28.81% to 96.70%), respectively, against the PCR assay. The positive and negative predictive values were 97.1% (95% CI: 85.03% to 99.52%) and 28.6% (95% CI: 8.57% to 58.08%). Genital mycoplasmas were detected in 71.4% (35/49) of samples with the Mycofast Revolution assay with 49% (24/49) being *Ureaplasma* spp. and 22.4% (11/49) mixed strains. The multiplex PCR assay had a positivity rate of 89.8% (44/49) for genital mycoplasmas; mixed strains were present in 51% (25/49) of samples, *Ureaplasma* spp. in 16.3% (8/49) and *M. hominis* in 22.4% (11/49) of samples. **Conclusions** There was a fair agreement (κ=0.319) between the Mycofast Revolution assay and the mPCR assay. With the high prevalence rates of genital mycoplasmas, fast and efficient diagnostic methods are imperative to treat infections and minimise complications. The Mycofast Revolution assay is

simple to use, has a short turn-around time and interpretation of results are straightforward. This assay circumvents common problems experienced with conventional culture and molecular methods in diagnostic laboratories where skilled personnel are limited and can be used as an alternative diagnostic assay.

Keywords: *Mycoplasma hominis*, *Ureaplasma* spp., Mycofast, Antimicrobial susceptibilities, multiplex PCR assay

3.1 Background

Genital mycoplasmas, including *Mycoplasma genitalium, M. hominis* and *Ureaplasma* spp. are potentially pathogenic bacteria that frequently colonise the genitourinary system of sexually active individuals [1]. Infections by these bacteria can lead to genital infections as well as undesirable sequelae during pregnancy [2,3]. The challenge of conventional methods to diagnose mycoplasmas forces researchers to investigate more sensitive, reliable and rapid alternatives. Susceptibility testing becomes prominent in the background of widespread antimicrobial resistance and topographical variation and must be incorporated in these testing systems.

Bacterial resistance to routine antimicrobial agents is a growing and worldwide problem. The lack of a rigid cell wall renders genital mycoplasmas innately resistant to antimicrobial agents, such as β-lactam antibiotics and vancomycin [4]. General treatment options include agents like tetracyclines and fluoroquinolones [5]. Fluoroquinolone antimicrobial agents can be used to treat genital mycoplasma infections caused by strains that are resistant to agents, such as the tetracycline agent doxycycline [6]. Agents that are frequently used include ofloxacin, ciprofloxacin, levofloxacin, gemifloxacin and moxifloxacin [7]. Moxifloxacin is a more recent quinolone, which has the highest *in vitro* activity against genital mycoplasmas [7]. These agents interact with the DNA gyrase and topoisomerase IV of bacteria [8]. Accordingly, fluoroquinolone resistance is associated with mutations in the *gyr*A and *gyr*B genes and the *par*C and *par*E genes [9]. Tetracyclines and fluoroquinolones are the drugs of choice, yet these agents are contraindicated in pregnancy [10,11]. During pregnancy, macrolides like erythromycin are often used [1,11].

Strains of *M. hominis* have natural resistance to C14 and C15 macrolides (e.g. clarithromycin, erythromycin, azithromycin and roxithromycin), while *Ureaplasma* spp. are resistant to lincosamides like clindamycin [12,13]. Resistance of *Ureaplasma* spp. to macrolides is widely reported and is associated with mutations in the 23S rRNA gene [14,15]. Tetracycline resistance is found in no less than 10% of *Ureaplasma* strains and approximately 40% of these resistant strains demonstrate cross-resistance to erythromycin [16]. Increased resistance to tetracyclines in *Ureaplasma* spp. and *M. hominis* is associated with the presence of the moveable *tet*(M) genetic element, the solitary tetracycline resistance mechanism, which renders ribosomes resistant to this agent [17,18].

Phenotypic and genotypic methods for the identification of mycoplasmas are available. Culture is still regarded as the gold standard for the detection of recoverable bacteria like *M. hominis* and *Ureaplasma* spp.; however, a low sensitivity when compared to polymerase chain reaction (PCR) assays has been reported [19,20]. Culture is labour intensive and time consuming as it requires the use of an enrichment broth for up to seven days, followed by sub-culturing on solid media. Analytical sensitivities in the range of 60% are only obtained in skilled laboratories and identification is restricted to the genus level. The development of commercially available diagnostic assays, which are based on liquid broth cultures provide easy to use and faster alternatives to conventional culture methods for the detection of genital mycoplasmas [21]. The difficulty of laboratory culture methods to isolate *M. genitalium* complicates antimicrobial susceptibility testing [22]. There is currently no approved and commercially available diagnostic assay for the detection of *M. genitalium*; detection is mainly done by nucleic acid amplification tests (NAATs) [23].

The new commercially available Mycofast Revolution (ELiTech Diagnostic, France) assay is a CE approved assay, which provides easy identification and enumeration of *M. hominis* and/or *Ureaplasma* spp. within 24 h to 48 h [24]. Antimicrobial susceptibility testing is performed against five antimicrobial agents that include levofloxacin, moxifloxacin, erythromycin, clindamycin and tetracycline [24]. The Mycofast Revolution assay is a liquid method based on the ability of *Ureaplasma* spp. and *Mycoplasma hominis* to metabolize urea and arginine, respectively and consists of 20 wells that are pre-coated with a dehydrated culture medium (foal serum, yeast extract, cysteine, arginine, urea, phenol red and antibiotics) and contains a single broth with antimicrobials for transport and preservation of genital mycoplasmas (UMMt) (ELiTech Diagnostic, France). The Mycofast Revolution assay includes an additional screening tray, which can be used prior to inoculation to differentiate between positive and negative specimens and is much more cost-effective. The screening tray and test trays allow the detection of genital mycoplasmas at concentrations $\leq 10^3$ colour change units per millilitre (ccu/ml) and $>10^3$ ccu/ml, respectively.

Other commercially available diagnostic assays that are similar with regards to genital mycoplasma identification, antimicrobial susceptibility testing, turn-around time and ease of use include the Mycoplasma Duo kit (Sanofi Diagnostics Pasteur, France), the Mycoview (Ivagen) and MycoIST2 (BioMérieux) test kits [14,25]. The advantage of the Mycofast

Revolution assay is that antimicrobial susceptibility testing is performed on different antimicrobial agents with specific minimum inhibitory concentrations (MICs) as defined by the 2011 Clinical and Laboratory Standards Institute (CLSI) guidelines. Antimicrobial susceptibility testing is performed against five antimicrobial agents that include levofloxacin, moxifloxacin, erythromycin, clindamycin and tetracycline [24].

Molecular methods, such as PCR assays are reported to be more sensitive for diagnostic purposes than culture [26]. Waites *et al.* [26] indicated that PCR-positive, culture-negative specimens are likely to represent true positives due to the much higher sensitivity. Genotypic methods also allow for speciation, which is a limitation of culture [26]. Other advantages include rapid detection as well as that these assays do not rely on the viability of the bacterium for detection [27]. Furthermore, when using a multiplex PCR (mPCR) assay, the detection of more than one target in a single reaction is possible and this can simplify the workflow [28].

The new commercially available Mycofast Revolution assay may have the potential to be used as a simplified and cost effective method to diagnose genital mycoplasmas. The purpose of the study was to compare the Mycofast Revolution assay with an mPCR assay for the detection of genital mycoplasmas from clinically collected vaginal specimens.

3.2 Methods

The study was conducted at the Department of Medical Microbiology, University of Pretoria from October 2012 to November 2012. Ethical approval was obtained from the Student Research Ethics Committee of the University of Pretoria prior to commencement of the study (Approved protocol number: S6/2012). The study population included pregnant women attending the antenatal clinic at a tertiary academic hospital in Pretoria, Gauteng, South Africa. The inclusion criteria of participants in this study were pregnant women attending the antenatal clinic who were older than 18 years and who gave written informed consent.

Two self-collected vaginal swabs (Copan Diagnostics, Inc, Italy) were obtained from fifty pregnant women. This was a convenient sample size to use and was not statistically calculated. The order in which the swabs were obtained was alternated between patients. After collection, a dry Rayon swab was used to seed 3 ml transport (UMMt) medium of the

Mycofast Revolution assay; the second swab (a flocked nylon swab) was inoculated into 1 ml of modified Amies transport medium (Copan Diagnostics, Inc, Italy) and used for PCR analysis. Inoculated media and reagents used were stored at 2ºC to 8ºC, whereas consumables were stored at room temperature (25ºC). The inoculated modified Amies transport medium was stored at -20ºC until DNA extraction was performed (within ±2 weeks of specimen collection). Extracted DNA was stored at -20ºC until PCR analysis (done within ±1 week after DNA extraction).

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 37ºC ± 1ºC 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 2ºC to 8ºC was used to inoculate the Complement Mycofast Revolution tray. Wells 1 to 20 were filled with 100 µl of seeded UMMt medium, wells 6 to 7 filled with an additional 50 µl of S.Mh and all the wells were covered with two drops of mineral oil. The tray was incubated (Vacutec, South Africa) at 37ºC ± 1ºC for 24 h (maximum 48 h in all cases) and after incubation observed for colour changes similar to that of the screening tray. *Mycoplasma hominis* (MH) identification wells contained erythromycin to inhibit the growth of *Ureaplasma* spp., while the UU wells contained lincomycin to inhibit the growth of *M. hominis*.

The specific breakpoints indicating susceptibility (S)/resistance (R) for *Ureaplasma* spp. are as follow [24]: levofloxacin S≤2, R≥4; moxifloxacin S≤2; erythromycin S≤8, R≥16; tetracycline S≤1, R≥2. The breakpoints for *M. hominis* are as follow: levofloxacin S≤1, R≥2; moxifloxacin S≤0.25; clindamycin S≤0.25, R≥0.5; tetracycline S≤4, R≥8. Strains were regarded as resistant when growth was inhibited by the higher critical concentration of the antimicrobial agent, but not the lower critical concentration or when growth was not inhibited by either the higher or lower critical concentrations of the antimicrobial agents.

Specimens were subjected to a human β-globin PCR assay to serve as an internal control and monitor possible PCR inhibitors. The mPCR assay used was adapted from the method by Stellrecht *et al*. [29]. This method was previously compared to the gold standard (culture on A7 agar) for genital mycoplasma identification and showed good sensitivity, specificity and positive and negative predictive values (87%, 96%, 94% and 93%, respectively) for the detection of genital mycoplasmas [29]. The mPCR assay was conducted with primers targeting genes specific for *M. genitalium, M. hominis*, *U. parvum* and *U. urealyticum* [29]. Oligonucleotide primers were synthesised by Inqaba Biotechnical Industries, South Africa. The mPCR assay was validated with AmpliRun *Mycoplasma genitalium* DNA control (Vircell SL, Spain), a positive *M. hominis* specimen isolated with A2 agar and reference strains ATCC 27813 (*U. parvum*) and ATCC 27619 (*U. urealyticum*).

Statistical analysis was performed using the PCR assay as the gold standard to calculate the sensitivity, specificity, positive predictive value and negative predictive value of the Mycofast Revolution assay. The positivity rates of both assays were determined and the agreement between the two methods was determined by the kappa (κ) statistic. The κ value, a measure of test reliability, was interpreted as follows: < 0.2, poor; 0.21 to 0.4, fair; 0.41 to 0.6, moderate; 0.61 to 0.8, good; ≥ 0.81, excellent [30].

3.3 Results

A total of 49 samples were included in this study. Contamination was observed in one specimen (2%) that was excluded from the analysis. The number of specimens that tested positive and negative with the Mycofast Revolution (phenotypic) and the mPCR (genotypic) assays as well as the breakdown according to species are displayed in Table 3.1.

Genital mycoplasmas were detected in 71.4% (35/49) of samples with the Mycofast Revolution assay. Forty-nine percent (24/49) of cultures were positive for *Ureaplasma* spp., while none of the cultures were positive for only *M. hominis*. Mixed strains (*M. hominis* and *Ureaplasma* spp.) were present in 22.4% (11/49) of cultures. Mycoplasmas were not detected in 28.6% (14/49) of specimens. One sample was positive for genital mycoplasma species with the Mycofast Revolution assay but negative with the mPCR assay.

Ureaplasma spp. were resistant to levofloxacin and moxifloxacin in 42% (10/24) and 4% (1/24) of cases, respectively (Table 3.2). *Ureaplasma* spp. had susceptibilities of 25% (6/24) and 21% (5/24) to erythromycin and tetracycline, respectively. The resistance patterns for mixed isolates were similar to those of *Ureaplasma* spp., except for erythromycin and tetracycline to which 100% (11/11) of the isolates were resistant.

The mPCR assay detected genital mycoplasmas in 89.8% (44/49) of specimens. *Ureaplasma* spp. were detected in 16.3% (8/49), while *M. hominis* was detected in 22.4% (11/49) of specimens. Fifty-one percent (25/49) of specimens were positive for both *Ureaplasma* spp. and *M. hominis*. The mPCR assay results showed only 10.2% (5/49) of specimens to be negative. About 82% (9/11) of the 11 cases that were culture positive for *M. hominis* were also positive with the mPCR assay.

Statistical analysis, when considering the mPCR assay as the gold standard, showed a sensitivity and specificity of 77.3% (95% CI: 62.15% to 88.51%) and 80% (95% CI: 28.81% to 96.70%), respectively for the Mycofast Revolution assay to detect genital mycoplasmas. The positive and negative predictive values were 97.1% (95% CI: 85.03% to 99.52%) and 28.6% (95% CI: 8.57% to 58.08%) respectively. The kappa statistic was 0.319.

3.4 Discussion

This study is the first to compare the Mycofast Revolution commercial assay against an mPCR assay for the detection of genital mycoplasmas from clinical specimens in South Africa. There was a fair agreement (κ=0.319) between the results of the phenotypic and genotypic methods. The Mycofast Revolution assay showed a high sensitivity and specificity, of 77% and 80% respectively, considering it only detects viable bacteria. However, this contributed to a low negative predictive value (28.6%) when the mPCR assay was considered the gold standard.

The positivity rates reported in this study are high (71% for the Mycofast Revolution and 91.8% for the mPCR assays). A study by Bayraktar *et al*. [31] in pregnant women, including symptomatic and asymptomatic control patients, reported a prevalence of 29% for genital mycoplasmas. An Iranian study (2009) reported a prevalence of 37% in outpatient women with clinical vaginitis [32]. Both of these studies identified genital mycoplasmas with the

commercially available Mycoplasma IST-2 kit. Govender *et al*. screened low-risk antenatal patients in South Africa at their first antenatal visit (16 to 23 weeks' gestation) for mycoplasmas at two different time frames (2003 and 2005) [3]. This research group used an mPCR assay and documented prevalence rates of genital mycoplasmas of almost 80% and around 40% in 2003 and 2005, respectively [3]. Nonetheless, the type of assay may have an effect on the accurate detection of genital mycoplasmas, depending on the growth factors and antimicrobial agents included in the media of the commercial assay.

A higher detection rate was observed for *Ureaplasma* spp. (detected alone in 24% of specimens) compared to *M. hominis* (never detected alone) with the Mycofast Revolution assay. The opposite was true for the mPCR assay with *M. hominis* being detected more frequently (detected alone in 22.4% of specimens, 6.1% more than the single detection of *Ureaplasma* spp.). The reason for the higher detection rate by the mPCR assay could be ascribed to specimens containing a low concentration of bacteria that were not detected after 48 h with the Mycofast Revolution assay. In such cases, the presence of genital mycoplasmas may possibly be colonisation instead of infection as the Mycofast Revolution assay is designed to detect whether the pathological threshold was reached.

A limitation of the Mycofast Revolution assay is that a low concentration of *M. hominis* may result in random wells to turn positive. Nonetheless, a specimen would only be regarded as positive if the identification wells are positive and the pathological thresholds are reached. The Mycofast Revolution assay does not distinguish between the species, *U. parvum* and *U. urealyticum* and analysis with molecular methods is needed for speciation. The specimen which tested positive with the Mycofast Revolution assay but negative with the PCR assay was neither re-extracted nor repeated with PCR. The fact that *M. hominis* was never detected alone may result in a low sensitivity and negative predictive value for the Mycofast Revolution assay to detect this bacterium. The antimicrobial susceptibilities of *M. hominis* were also masked by the resistance of *Ureaplasma* spp. and therefore the antimicrobial susceptibility profiles of the mixed isolates could not be extrapolated to *M. hominis.*

Although PCR assays have the advantage of being sensitive, it remains costly and is dependent on skilled personnel. The inoculation and handling of the Mycofast Revolution assay do not require skilled personnel and the results are easy to interpret. In addition, the

Mycofast Revolution assay allows quantitation of the number of mycoplasmas present and gives an indication of colonisation or infection. Despite the lower observed sensitivity of the Mycofast Revolution assay compared to the mPCR assay, the main advantage of the Mycofast Revolution assay is that it tests the activity of a variety of new antimicrobial agents against genital mycoplasmas with updated MICs as defined by the 2011 CLSI guidelines [24]. This may reduce the cost of antimicrobial surveillance and renders the Mycofast Revolution assay of clinical importance in the era of increasing antimicrobial resistance. The Mycofast Revolution assay may be an acceptable assay to use in routine diagnostic laboratories in South Africa where resources are limited. It may be used as an alternative in laboratories where insensitive conventional culture methods are used.

The difference in the findings between the two assays can be ascribed to numerous factors, including different bacterial loads on the different swabs, the viability of bacteria and the difference in analytical sensitivities of the two assays. A limitation of the study was that an additional molecular assay was not used to resolve the discrepancies between the two assays.

3.5 Conclusions

The Mycofast Revolution assay could be considered as a cost-effective alternative to conventional culture methods for the rapid detection of genital mycoplasmas. The assay may allow laboratory personnel to provide the clinician with a result in a short period (± 48 hours) of time together with antimicrobial susceptibility data. Antimicrobial susceptibility patterns are vital as successful treatment will depend on the early administration of effective antimicrobial agents. In pregnant women it is particularly important to reduce these infections to prevent adverse pregnancy outcomes.

Competing interests

None to declare. The authors would like to thank Separation Scientific for supplying the Mycofast Revolution kits used in this study. The authors would also like to thank the University of Pretoria, the Medical Research Council (South Africa) and the National Health Laboratory Service (NHLS) for financial assistance received.

Authors' contributions

Mathys J Redelinghuys was involved in concept design, laboratory work as well as writing of the manuscript. Marleen M Kock, Marthie M Ehlers and Andries W Dreyer were involved in concept design of the study as well as critical review of the manuscript. Hennie A Lomaard was involved in concept design of the study as well as overseeing the logistics of sample collection.

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TABLES AND FIGURES

Table 3.1: Results of *M. hominis* and *Ureaplasma* spp. after the Mycofast Revolution and mPCR assay analyses (n=49)

Assay	*M. hominis* (MH)[1] No (%)	*Ureaplasma* spp. (UU + UP)[1] No (%)	Mixed isolation: MH + (UU + UP) No (%)	Negatives No (%)	Total
Mycofast Revolution	0	24 (49)	11 (22.4)	14 (28.6)	49
PCR	11 (22.4)	7 (14.3)	26 (53)	5 (10.2)	49

[1]Where MH is *M. hominis*, UP is *U. parvum* and UU is *U. urealyticum*

Table 3.2: The distribution (%) of *Ureaplasma* spp. and *M. hominis* at different breakpoints of antimicrobial agents (n=49)

	Levofloxacin			Moxifloxacin		Erythromycin		Clindamycin		Tetracycline			
	1[1]	**2**	**4**	**0.25**	**2**	**8**	**16**	**0.25**	**0.5**	**1**	**2**	**4**	**8**
Sensitive (S) / Resistant (R)	**S**	**R**	**R**	**S**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
***Ureaplasma* species (n=24)**	50	29	13	88	4	71	4	0	100	17	25	4	33
***Ureaplasma* species and *M. hominis* (n=11)**	73	18	9	82	9	9	91	0	100	9	18	9	64

[1]The breakpoints in µg/mL according to the CLSI guidelines[24]

Table 3.3: The overall number of specimens that tested positive and negative with the mPCR and Mycofast Revolution assays

		Multiplex PCR assay		
		Positive	**Negative**	**Total**
Mycofast Revolution assay	**Positive**	34	1	35
	Negative	10	4	14
	Total	44	5	49

Table 3.4: The number of specimens that tested positive and negative for *Ureaplasma* spp. with the mPCR and Mycofast Revolution assays

		Multiplex PCR assay		
		Positive	**Negative**	**Total**
Mycofast Revolution assay	**Positive**	31	4	35
	Negative	2	12	14
	Total	33	16	49

Table 3.5: The number of specimens that tested positive and negative for *M. hominis* with the mPCR and Mycofast Revolution assays

		Multiplex PCR assay		
		Positive	**Negative**	**Total**
Mycofast Revolution assay	**Positive**	9	4	11
	Negative	28	10	38
	Total	37	12	49

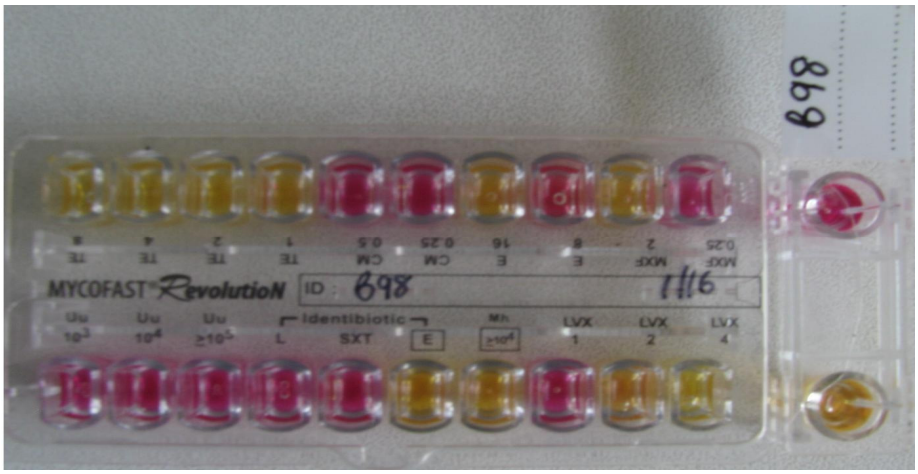

Figure 3.1: **Mycofast Revolution test (left) and screening (right) trays. The screening tray shows a positive identification result for *Ureaplasma* spp. (top, red) and a negative identification result for *M. hominis* (bottom, yellow), corresponding to the identification on the test tray (positive, red colour for *Ureaplasma* spp. in the L and SXT wells and a negative, yellow colour for *M. hominis* in the E well)**

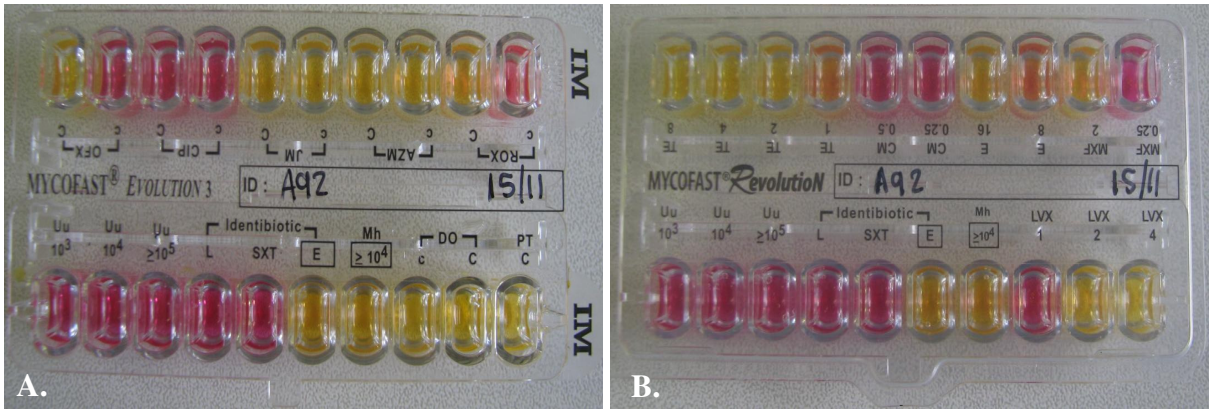

Figure 3.2: **Two different versions of the commercial Mycofast series. Figure (A) displays the Mycofast Evolution 3 assay, an older version and (B) displays the Mycofast Revolution assay, the latest version of the Mycofast series. Both assays contain *Ureaplasma* spp. enumeration wells (10^3, 10^4 and $\geq 10^5$), identification wells (L and SXT for *Ureaplasma* spp. and E for *M. hominis*), a *M. hominis* enumeration well ($\geq 10^4$) and antimicrobial coated wells with different antimicrobial agents**

CHAPTER 4

Antimicrobial susceptibilities of *Ureaplasma* species and *Mycoplasma hominis* in pregnant women: an experimental study

The editorial style of the British Journal of Obstetrics and Gynaecology was followed in this chapter

Abstract

Objective To investigate the antimicrobial susceptibility patterns of genital mycoplasmas in pregnant women. **Design** This was an experimental study with qualitative data analysis. **Setting** Antenatal clinic of a tertiary academic hospital in Pretoria, Gauteng, South Africa. **Population** Self-collected vaginal swabs were obtained from 96 pregnant women **Methods** Specimens were screened with the Mycofast Revolution assay for the presence of *Ureaplasma* species and *Mycoplasma hominis*. The antimicrobial susceptibility to levofloxacin, moxifloxacin, erythromycin, clindamycin and tetracycline were determined at various breakpoints. A multiplex polymerase chain reaction (mPCR) assay was used to speciate *Ureaplasma* positive specimens as either *U. parvum* or *U. urealyticum*. **Main outcome measures** High levels of tetracycline and erythromycin resistance with emerging fluoroquinolone resistance in genital mycoplasmas. **Results** Seventy-six percent (73/96) of specimens contained *Ureaplasma* spp., while 39.7% (29/73) of *Ureaplasma* positive specimens were also positive for *M. hominis*. Susceptibilities of *Ureaplasma* spp. to levofloxacin and moxifloxacin were 59% (26/44) and 98% (43/44), respectively. Mixed isolates (*Ureaplasma* species and *M. hominis*) were highly resistant to erythromycin and tetracycline (both 97% resistance). Resistance of *Ureaplasma* spp. to erythromycin was 80% (35/44) and tetracycline resistance was detected in 73% (32/44) of *Ureaplasma* spp. Speciation indicated that *U. parvum* was the predominant *Ureaplasma* spp. conferring antimicrobial resistance. **Conclusions** Treatment options for genital mycoplasma infections are becoming limited. More elaborative studies are needed to elucidate the diverse antimicrobial susceptibilities found in this study when compared to similar studies. These studies may evaluate routine screening of antimicrobial susceptibility patterns in similar settings as a measure to reduce complications in pregnant women, the foetus and the neonate.

Keywords: Pregnant women, *Ureaplasma* spp., *Mycoplasma hominis*, antimicrobial susceptibilities

4.1 Introduction

Mycoplasma hominis and *Ureaplasma* spp., including *U. parvum* and *U. urealyticum,* are commonly found in the vaginal milieu of up to 80% of pregnant and non-pregnant women[1]. Although controversial, some studies suggest that during pregnancy, mycoplasmas may ascend from the vagina/cervix, penetrate the chorioamnion and invade the amniotic cavity to cause complications, such as preterm birth and spontaneous abortion[2,3]

Mycoplasmas display inherent resistance to beta-lactams and glycopeptides (e.g. vancomycin) due to the absence of a cell wall[4]. Although macrolides are often the drugs of choice for treating these infections, *M. hominis* is intrinsically resistant to C14 and C15 macrolides (e.g. erythromycin and azithromycin)[5]. *Ureaplasma* species also have natural resistance to lincosamides (e.g. clindamycin)[6]. Observed resistance to macrolides is associated with mutations in the 23S rRNA gene[7,8], while resistance to tetracyclines is associated with the presence of the moveable *tet*(M) genetic element[9,10].

The administration of antimicrobial agents to pregnant women with preterm rupture of the membranes may extend the gestation period and decrease the risks of associated complications and neonatal infections[11]. The antimicrobial agent of choice should be considered carefully as some agents are teratogens, i.e. the agent can cause malformation or functional damage to an embryo or foetus, or may have toxic effects on the neonate[12]. Macrolides are often empirically used[13] because tetracyclines and fluoroquinolones are contraindicated in pregnancy[11,14]. However, the amniotic sac is not effectively penetrated by erythromycin and ureaplasmas are not eradicated from the vagina or cervix by this agent[4]. Newer macrolides (e.g. azithromycin and clarithromycin) allow for better tolerability and once daily dosing can increase compliance[4,15]. Treatment with azithromycin is equally successful compared to erythromycin with fewer side effects[16,17].

To perpetuate the effective use of antimicrobial agents, the antimicrobial activities of such agents need to be monitored frequently. The Mycofast Revolution assay allows for detection, identification and antimicrobial susceptibility testing of genital mycoplasmas within 48 hours. However, identification with this assay is limited to *M. hominis* and the *Ureaplasma* genus. The speciation of genital mycoplasmas can be achieved by the use of sensitive and rapid molecular methods, such as PCR assays[8]. Speciation of bacteria may assist in elucidating the

pathogenesis of specific medical conditions[19]. The purpose of this study was to investigate the antimicrobial susceptibility patterns of genital mycoplasmas in pregnant women attending antenatal care.

4.2 Materials and Methods

This was an investigative study that included pregnant women attending the antenatal and maternal and foetal unit (MAFU) clinics of a tertiary academic hospital in Pretoria, South Africa from October 2012 to January 2013. 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 S6/2012) and preceded experimental work. Experimental work was conducted at the Department of Medical Microbiology, University of Pretoria.

Self-collected vaginal swabs (Copan Diagnostics, Inc, Italy) were obtained from 96 women and were tested for the presence of *Ureaplasma* species and *M. hominis*. Identification, enumeration and antimicrobial susceptibilities were determined with the Mycofast Revolution assay (ELiTech Diagnostic, France) as indicated by the manufacturer. Briefly, the swab was used to seed the UMMt transport medium of which 100 µl was added to the *U. urealyticum* (UU) and *M. hominis* (MH) wells of the Mycofast Screening Revolution tray. Additionally, 50 µl of MH supplement (S.Mh) was 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 37ºC for 24 h. After 24 h of incubation, an orange or red colour change indicated the presence of *Ureaplasma* species and/or *M. hominis*. A yellow colour in the wells marked the absence of mycoplasmas. In the case of a positive screening test, the remainder of the seeded UMMt that was stored at 4ºC to 8ºC was used to inoculate the Complement Mycofast Revolution tray. One hundred microliters of UMMt medium was dispensed into wells 1 to 20, 50 µl of S.Mh was dispersed into wells 6 and 7. All the wells were covered with two drops of sterile mineral oil. The tray was incubated (Vacutec, South Africa) at 37ºC ± 1ºC for 24 h (maximum 48 h in all cases) and the presence or absence of colour changes at breakpoints, specified on the inoculation trays, indicated resistance or susceptibility to each antimicrobial agent.

Genital mycoplasma strains were regarded as sensitive when growth was inhibited by the higher and lower critical concentrations of the antimicrobial agent. Strains were regarded as resistant when growth was inhibited by the higher critical concentration of the antimicrobial agent, but not the lower critical concentration or when growth was not inhibited by either the higher or lower critical concentrations of the antimicrobial agents. The specific breakpoints indicating susceptibility (S) or resistance (R) for *Ureaplasma* species are as follow[20]: levofloxacin S ≤2, R ≥4; moxifloxacin S ≤2; erythromycin S ≤8, R ≥16; tetracycline S ≤1, R ≥2. The breakpoints for *M. hominis* are as follow: levofloxacin S ≤1, R ≥2; moxifloxacin S ≤0.25; clindamycin S ≤0.25, R ≥0.5; tetracycline S ≤4, R ≥8.

A multiplex PCR (mPCR) assay was used to determine the *Ureaplasma* species detected with the Mycofast Revolution assay. This assay was performed as described by Stellrecht *et al*.[2] and was validated with reference strains ATCC27813 (*U. parvum*) and ATCC27619 (*U. urealyticum*). The multiple-banded antigen (MBA) gene of *Ureaplasma* served as the target gene. The amplified products were subjected to gel electrophoresis at 100 V for 1 h on a 2% (m/v) MetaPhor agarose gel (Lonza, USA) in 1X TBE buffer [45 mM Tris-borate (pH 8.0) (Sigma Chemical co, USA), 1 mM EDTA (Promega, Madison, USA)]. A 50 kb molecular marker (Fermentas, Thermo Scientific, USA) was used to identify band sizes, which were 403 bp and 448 bp in size for *U. parvum* and *U. urealyticum*, respectively[2].

4.3 Results

Ureaplasma species were detected in 76% (73/96) of specimens and 39.7% (29/73) of *Ureaplasma* positive specimens were also positive for *M. hominis*. *Mycoplasma hominis* was not detected alone. The growth of *Ureaplasma* species and *M. hominis* at various breakpoints of antimicrobial agents are displayed in Table 1. The presence of both *Ureaplasma* species and *M. hominis* in a single specimen were denoted as mixed isolates.

Ureaplasma species had susceptibilities of 59% (26/44) and 98% (43/44) to the newer fluoroquinolones namely levofloxacin and moxifloxacin, respectively. Eighty percent (35/44) of *Ureaplasma* strains were resistant to erythromycin, whereas resistance to tetracycline was detected in 73% (32/44). Greater resistance was observed for mixed isolates against erythromycin and tetracycline [both 97% (28/29)] at higher critical concentrations. The susceptibility patterns of the mixed isolates to levofloxacin were similar to the cases where

only *Ureaplasma* species were detected, while mixed isolates were more resistant to moxifloxacin (Figure 4.1).

Results of the mPCR assay indicated that 95% (42/44) of the *Ureaplasma* positive specimens contained only *U. parvum,* while 5% (2/44) contained both *U. parvum* and *U. urealyticum*. None of the specimens that were positive for *Ureaplasma* species had only *U. urealyticum* present.

4.4 Discussion

The high level of antimicrobial resistance to erythromycin and tetracycline that was observed for mixed isolates may be attributed to *M. hominis*. The antimicrobial resistance was significantly higher than when only *Ureaplasma* species were detected (97% vs 80% for erythromycin, p=0.0396; and 97% vs 73% for tetracycline, p=0.0101). Erythromycin resistance of mixed isolates is comparable to the results of Domingues *et al*.[21] who documented erythromycin resistance in 90.7% of *Ureaplasma* species and *M. hominis* positive cases. Kechagia and colleagues[5] reported a 100% resistance (33% intermediate and 67% complete resistance) rate to erythromycin for mixed isolates. In the present study, tetracycline resistance of the mixed isolates was higher (97%) when compared to similar studies (14% to 57%)[5,13].

Resistance of *Ureaplasma* species to erythromycin (80%) corresponded to the 83% found by Kechagia and co-workers[5]; however, the researchers reported that 87.4% of ureaplasmas were susceptible to tetracycline, while the present study found only 27% of *Ureaplasma* isolates to be susceptible to tetracycline. Another study reported a resistance of only 17.2% to erythromycin and 19% to tetracycline[13]. Bayraktar and colleagues[1] reported a 100% tetracycline susceptibility of *U. urealyticum* isolated from pregnant women in Turkey. It is worth noting that these studies reported lower tetracycline resistance rates specifically for *U. urealyticum*.

Results of the mPCR assay indicated that 95% (42/44) of the *Ureaplasma* positive specimens contained only *U. parvum,* while 5% (2/44) contained both *U. parvum* and *U. urealyticum*. *Ureaplasma parvum* was the principal species contributing to antimicrobial resistance. Similar results were found by Povlsen *et al*.[22] who investigated genital mycoplasmas in

pregnant women with singleton pregnancies and reported that approximately 90% of the 280 *Ureaplasma* positive specimens contained *U. parvum* and 3% contained both *U. parvum* and *U. urealyticum*. After speciation of the *Ureaplasma* positive specimens, it was discovered that *U. parvum* strains conferred resistance to fluoroquinolones (levofloxacin and moxifloxacin) and macrolides (erythromycin). These results are similar to that of Govender *et al*.[23] who reported fluoroquinolone and erythromycin resistance in *U. parvum* strains from South Africa.

Zhu and colleagues[24] reported antimicrobial susceptibilities of 10.65% and 31.27% to levofloxacin for mixed isolates and *U. urealyticum*, respectively. The present study found genital mycoplasmas to be more susceptible to levofloxacin with susceptibilities of 59% and 58% for *Ureaplasma* species and mixed isolates, respectively.

The susceptibilities of genital mycoplasmas to antimicrobial agents differ by geographical region[13]. Govender and co-workers[23] amplified and sequenced *tet*(M) genes in tetracycline resistant ureaplasmas and found the *tet*(M) genes of strains isolated in the Cape Town region of South Africa to be diverse, while the *tet*(M) genes isolated from tetracycline resistant ureaplasmas in the Pretoria region were frequently similar in structure[23]. A possible reason for the difference in antimicrobial resistance in the present study and reports from various countries may be the result of different antimicrobial-usage guidelines, which led to the resistance of bacterial strains to different antimicrobial groups[5]. Additional variables contributing to the difference in resistance may include the population studied, the study period or the kits used for specimen processing and analyses[25]. The establishment of common guidelines for the treatment of genital mycoplasma infections is complex and effective treatment depends on the antimicrobial susceptibilities of genital mycoplasmas in a specific region[5,26].

Roberts[27] proposed strategies to preserve the use of current agents like the MLSKO group and minimise resistance to antimicrobial agents. Strategies include the development of (i) new classes of antimicrobial agents, (ii) updated derivatives of currently used antimicrobial agents and (iii) therapies other than antimicrobial agents[27]. The use of two different antimicrobial agents for treatment instead of one agent may aid in prolonging the efficacy of such agents[27]. Antimicrobial agents need to be screened for sensitivity on a regular basis and when used as

treatment, these agents should be administered as early as possible for good pregnancy outcomes[1,26]. However, effective resources are required to constantly monitor antimicrobial susceptibility profiles of such agents to ensure treatment success[27].

The detection of mycoplasmas with conventional culture methods is labour intensive and time consuming and is not routinely done in many diagnostic laboratories[1,25,26]. To circumvent the delays that may be experienced with the diagnosis of genital mycoplasma infections, the syndromic management of sexually transmitted infections (STIs) is a general treatment approach. A number of studies reported that the syndromic approach is not effective in reducing the prevalence of curable STIs in asymptomatic patients[28,29,30]. Nonetheless, the success of the syndromic management of STIs relies on up-to-date knowledge of the infectious agents causing specific syndromes and the antimicrobial susceptibilities of these agents[31]. The frequent use of erythromycin in pregnant women has allowed the surveillance of long-term effects of this antimicrobial agent. These include infantile hypertrophic pyloric stenosis[32], cardiac toxicity[33] and maternal hepatotoxicity[34]. There is not yet enough data available to know whether the risks of toxicity in neonates are similar with newer macrolide antimicrobial agents[4]. If the price of azithromycin decreases to an affordable level it may potentially replace erythromycin as a general treatment option in the future. Fluoroquinolones are classified as category C agents and the use of these agents in pregnancy is controversial[18]. The treatment options of genital mycoplasmas in pregnancy remains limited.

Monitoring of susceptibility patterns of genital mycoplasmas may assist with optimising treatment guidelines and overall improve therapeutic outcome. The Mycofast Revolution assay is an easy and effective way of evaluating the susceptibility of genital mycoplasmas to commonly used or potential antimicrobial agents.

Nonetheless, in this study the Mycofast Revolution assay had limited capability of providing antimicrobial susceptibility profiles specific for *M. hominis*. This was because in every instance this bacterium was isolated in culture, it was mixed with *Ureaplasma* spp. Another limitation of the study is that bacterial strains were not analysed for specific mutations where antimicrobial resistance was detected. 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. These studies can be performed in broader study populations, such as non-pregnant women, HIV positive women and possibly men.

4.5 Conclusion

Although the fluoroquinolones, especially moxifloxacin, remain the most effective against genital mycoplasmas, these agents are restricted to non-pregnant patients. Discrepant antimicrobial susceptibility results from different regions emphasise the importance of routine monitoring to ensure the efficacy of treatment and ultimately curb morbidity and mortality rates.

Competing interests

None to declare.

Authors' contributions

Mathys J Redelinghuys was involved in concept design, laboratory work as well as writing of the manuscript. Marleen M Kock, Marthie M Ehlers and Andries W Dreyer were involved in concept design of the study as well as critical review of the manuscript. Hennie A Lomaard was involved in concept design of the study as well as overseeing the logistics of sample collection.

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TABLES AND FIGURES

Table 4.1: The distribution (%) of *Ureaplasma* species and *M. hominis* at different breakpoints of antimicrobial agents (n=96)

	Levofloxacin			Moxifloxacin		Erythromycin		Clindamycin		Tetracycline			
	1[1]	2	4	0.25	2	8	16	0.25	0.5	1	2	4	8
Sensitive (S) / Resistant (R)	**S**	**R**	**R**	**S**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**
***Ureaplasma* species (n=44)**	52	30	11	93	2	70	9	0	100	25	16	2	30
***Ureaplasma* species and *M. hominis* (n=29)**	55	28	14	83	10	10	86	0	100	14	28	7	48

[1]The breakpoints in µg/mL according to the CLSI guidelines[19]

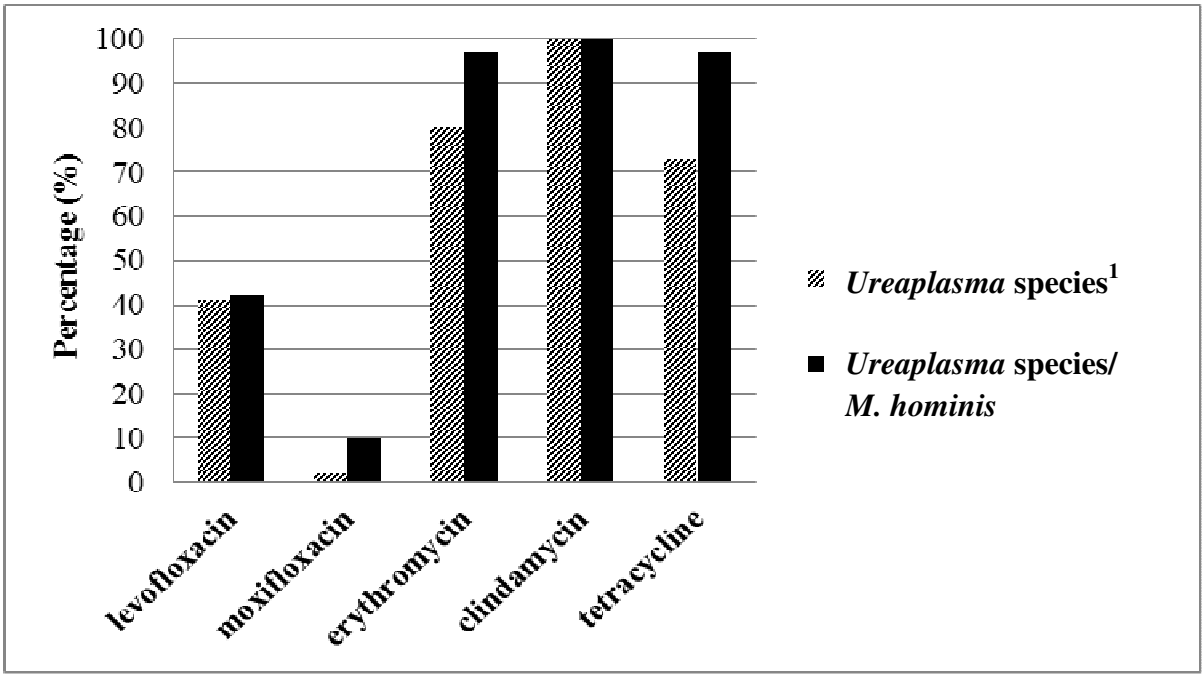

Figure 4.1: Antimicrobial resistance (%) of *Ureaplasma* spp. and *M. hominis* positive specimens to various antimicrobial agents. *Ureaplasma parvum* made up 95% of the *Ureaplasma* spp.

CHAPTER 5

The association between bacterial vaginosis and genital mycoplasmas in pregnant women in Pretoria, South Africa

The editorial style of the British Journal of Obstetrics and Gynaecology was followed in this chapter

Abstract

Background Bacterial vaginosis (BV) and genital mycoplasmas are reproductive tract infections (RTIs) that are associated with several infections and adverse pregnancy outcomes, such as pelvic inflammatory disease, preterm birth and pregnancy loss in affected women. Bacterial vaginosis is a dysbiosis, which is prevalent in 10% to 40% of pregnant women. Genital mycoplasmas are reported to colonise up to about 70% of sexually active women. The purpose of the study was to determine the association between BV and genital mycoplasmas. **Methods** Self-collected vaginal swabs were obtained from pregnant women with (high-risk) and without (low-risk) a history of at least one pregnancy loss. Bacterial vaginosis was diagnosed using the Nugent scoring system. The A2 agar medium and a multiplex PCR assay were used to detect genital mycoplasmas. The 140-kDa adhesion protein, 16S rRNA gene and the multiple-banded antigen genes were used as targets for the detection of *Mycoplasma genitalium, M. hominis, Ureaplasma parvum* and *U. urealyticum*, respectively. **Results** A total of 220 women were included in the study of which 48.2% (106/220) were considered high-risk and 51.8% (114/220) low-risk. The mean and median gestational age of current pregnancies were 26 weeks and most women [45.9% (101/220)] were in the second trimester of pregnancy. The prevalence of BV was 17.7% (39/220), whereas the prevalence of genital mycoplasmas ranged from 2.3% to 71.4%. Culture media failed to isolate genital mycoplasmas and was frequently contaminated with other bacterial species. Bacterial vaginosis was significantly (95% CI: 2.2256 to 13.7542; p=0.0002) detected in women with 1st trimester pregnancies (11/23), whereas only *M. hominis* was significantly (95% CI: 1.5916 to 8.0142; p=0.0020) isolated from HIV positive women (27/36). *Mycoplasma hominis* (95% CI: 1.4108 to 6.4025; p=0.0043) and *U. parvum* (95% CI: 1.0028 to 6.3739; p=0.0493) were associated with BV more often than were *M. genitalium* and *U. urealyticum.* Neither BV nor genital mycoplasmas were significantly present in either low- or high-risk women. **Conclusions** This study found that *M. hominis* and *U. parvum* are associated with

BV. Infections with these bacteria may be misdiagnosed because no routine screening is done in South Africa (SA). The low effectiveness of syndromic treatment to reduce the prevalence of asymptomatic RTIs necessitates improved diagnostic and treatment strategies. Additional studies are needed to investigate the detection and intervention of these infections as a preventative measure to minimise complications, such as undesirable pregnancy outcomes and decrease neonatal morbidity and mortality in this study population.

Keywords: Bacterial vaginosis, Genital mycoplasmas, PCR, Pregnancy, Reproductive health, Reproductive tract infections

5.1 Introduction

Bacterial vaginosis is the most common cause of vaginal discharge in women of childbearing age[1]. The prevalence of BV ranges from 10% to 40% during pregnancy[2]. Genital mycoplasmas, including *M. genitalium*, *M. hominis*, *U. parvum* and *U. urealyticum* may reach colonisation rates of up to 80% in women[3,4].

The association between BV and genital mycoplasmas is contentious; some studies support an association[5], whereas other studies do not[6]. *Mycoplasma hominis* and *U. urealyticum* are reported to be associated more with BV than is the case for other genital mycoplasmas[7,8,9]. Bacterial vaginosis and genital mycoplasmas are implicated in medical conditions, such as pelvic inflammatory disease (PID), preterm birth (PTB), pregnancy loss and post-partum and post-abortal sepsis[4,10,11].

Pregnancy loss can be classified as either early (before 12 weeks gestation) or late (after 12 weeks gestation)[12]. It is estimated that first trimester (<12 weeks) pregnancy loss may occur in up to 30% of all pregnancies (of which only 15% to 20% of cases are reported)[12,13]. Pregnancy loss in the second trimester, between 12 and 26 weeks, occurs less often than first trimester pregnancy loss and comprises <4% of pregnancy outcomes[12]. Pregnancy loss in the first and second trimesters is associated with infection of the amniotic membranes significantly more than third trimester loss[14,15]. This is especially true for loss after 36 weeks of gestation where infection is rare[16]. The reasons for decreased intrauterine infection with increasing gestation are unclear[15].

Genital mycoplasmas may travel from the vagina and penetrate the amniotic membranes to produce inflammation (chorioamnionitis), resulting in pregnancy loss[15,17]. This may be enhanced by BV, which favours ascending infections[18]. Genital mycoplasmas are the most frequently isolated bacteria in women who present with chorioamnionitis, being responsible for 45% of these cases[15,19]. Women with BV, whether symptomatic or asymptomatic, are significantly more likely to experience a late pregnancy loss[20]. Donders *et al.*[21] found that BV, especially when *G. vaginalis* or *M. hominis* and *U. urealyticum* are cultured, is associated with a five-fold increased risk of pregnancy loss. *Mycoplasma hominis* and *U. urealyticum* are reported to increase the risk for recurrent pregnancy loss[22]. *Ureaplasma parvum* has been linked with late pregnancy loss in colonised women[23]. Information on *M. genitalium* and

specific adverse pregnancy outcomes is limited. Labbe and colleagues[24] employed a polymerase chain reaction (PCR) assay and isolated *M. genitalium* from 6.2% of cervical specimens. This research group did not report any significant association between the presence of *M. genitalium* in the cervix and pregnancy loss[24].

The Nugent scoring system is the gold standard for the diagnosis of BV and is based on Gram-stained vaginal smears that are microscopically analysed[25,26]. Gram-stained smears are graded according to a combination of bacterial morphotypes present[25]. However, this system may lack sensitivity when *A. vaginae* is investigated because this bacterium is not readily detected by Gram-staining[27]. *Mycoplasma hominis* and *Ureaplasma* spp. are routinely detected by culture methods and a selective agar medium like A2 agar is used to distinguish between genera[7,28]. However, these methods are laborious and compared to PCR assays, lack sensitivity for mycoplasma isolation[29]. As a result of the fastidious growth of *A. vaginae* and *M. genitalium*, which complicates identification by culture methods, PCR-based identification assays have been developed[29,30]. Molecular methods, such as PCR assays (conventional and real-time) are sensitive and allow for rapid detection of infectious agents and do not require the use of viable organisms[31].

The purpose of the study was to determine the prevalence of bacterial vaginosis and genital mycoplasmas in pregnant women attending a tertiary academic hospital. The possible association of specific genital mycoplasmas with bacterial vaginosis was investigated.

5.2 Materials and Methods

5.2.1 Study setting and study population

This study was conducted at the Department of Medical Microbiology, University of Pretoria from July 2012 to March 2013. Prior to commencement of the study, ethical approval was obtained (protocol number S2/2012) from the Student Research Ethics Committee of the University of Pretoria. Specimens were obtained from pregnant women attending the antenatal and Maternal and Foetal Unit (MAFU) clinics of a tertiary academic hospital in Pretoria, Gauteng, South Africa. Women were divided into two groups according to underlying conditions. Women who were considered as having low-risk pregnancies included women with mainly diabetes, cardiac problems, epilepsy, asthma etc.; women with a history of one or more pregnancy loss(es), including a miscarriage, ectopic pregnancy and stillbirth

(all resulting in foetal death) were considered as having a high-risk pregnancy[32]. All the participants were older than 18 years of age and gave written informed consent prior to enrolment.

5.2.2 Specimen collection and processing

Two self-collected vaginal swabs (Copan Diagnostics, Inc., Italy), a dry Rayon swab and an eSwab (a flocked nylon swab containing 1 ml modified Amies transport medium), were obtained from every participant. The order in which the dry swab and eSwab were obtained was alternated between patients. The dry swab was used to make a smear on a glass slide (B&C, Germany) immediately after the swab was taken and left to air dry. Immediately after obtaining the specimen, the eSwab was used to seed 1 ml of Amies transport medium and was stored on ice. The swabs were kept on ice and transported to the laboratory within 1 to 4 h where it was processed for further analyses.

5.2.3 Culture of *M. hominis* and *Ureaplasma* spp.

The initial ninety-five specimens collected were diagnosed with culture methods. The growth of *M. hominis* and *Ureaplasma* spp. was enhanced with the use of Arginine broth (Diagnostic Media Products, South Africa) and U9 broth (Diagnostic Media Products, South Africa), respectively. Upon arrival at the laboratory, specimens were left for ± 15 min to reach room temperature (± 25ºC). An aliquot of 100 µl of the seeded Amies transport medium (Copan Diagnostics, Inc., Italy) was used to inoculate the Arginine broth (Diagnostic Media Products, South Africa) and U9 broth (Diagnostic Media Products, South Africa). The inoculated broths were incubated (Vacutec, South Africa) at 37°C with 5% CO_2 and were observed for any colour changes (from yellow/orange to red/pink) after 24 h of incubation. Broths were observed for colour changes for up to 7 days before being regarded as negative. Specimens that were positive with broth inoculation were subsequently grown on solid A2 selective *Mycoplasma* agar medium (Diagnostic Media Products, South Africa) by plating out 50 µl of positive broth and incubated (Vacutec, South Africa) at 37°C with 5% CO_2. Inoculated A2 selective *Mycoplasma* agar plates (Diagnostic Media Products, South Africa) were observed after 24 h and 48 h of incubation and depending on growth, incubated if necessary for up to 5 days. After incubation, growth was observed using an inverted microscope (American Optical Corporation, USA) with the 10X objective. The presence of

characteristic colonies was recorded: (i) *M. hominis*: round, "fried egg" colonies of 100 to 300 µm and (ii) *Ureaplasma* spp.: brownish-black "sea urchin" colonies of 10 to 50 µm[7].

5.2.4 Microscopy

Upon arrival at the laboratory, smears were fixed to slides by heating for 5 s using a Bunsen burner, Gram-stained and graded with the Nugent scoring system as described by Nugent *et al.*[25]. On a scale from zero to ten, a score of 0 to 3 was considered negative for BV, a score of 4 to 6 was considered intermediate (either in the process of developing BV or recovering from having BV) and a score of 7 to 10 was considered positive for BV (Appendix B). As a quality control measure, all the slides were re-read at a later stage and were also graded by a second examiner. Some slides were also compared to previously graded slides that served as controls to confirm the Nugent score.

5.2.5 Molecular detection of genital mycoplasmas

5.2.5.1 DNA extraction from modified Amies transport medium

Bacterial DNA was isolated from the vaginal swabs with the ZR Fungal/Bacterial DNA kit (Zymo Research, USA) according to the manufacturer's instructions (Appendix B). A volume of 150 µl seeded Amies transport medium (Copan Diagnostics, Inc, Italy) was used to extract bacterial DNA. One hundred microliters of ultra-pure DNA was eluted and stored at -20ºC until further analysis.

5.2.5.2 Amplification of human β-globin gene

Each sample was subjected to an internal positive control β-globin PCR assay to assure that amplifiable DNA was successfully extracted from the specimen, to monitor for PCR inhibitors and to exclude false negative results[22,33]. All β-globin gene PCRs were performed using the TaKaRa ExTaq PCR kit (Takara Biotechnology, Japan) in a Gradient MasterCycler (Eppendorf, Hamburg, Germany). The oligonucleotide primer sequences used were obtained from Martin *et al.*[34] (Table 5.1).

Amplification was performed in 50 µl reactions each containing 25 µl of 2X EmeraldAmp GT PCR Master Mix (Takara Biotechnology, Japan), 0.5 µl of each primer for a final concentration of 0.2 µM, 5 µl (< 500 ng) of extracted DNA as template and nuclease-free water. The amplification programme comprised an initial denaturation step at 95°C for 15

min, 40 cycles of three steps including denaturation at 95°C for 30 s, annealing at 56°C for 45 s and extension 72°C for 1 min, followed by a final extension step at 72°C for 7 min. The PCR products were separated on a 1% (m/v) agarose gel (Lonza, USA) in 1X Tris-borate EDTA (TBE) buffer [45 mM Tris-borate (pH 8.0) (Sigma Chemical co, USA), 0.5 M EDTA (Promega, Madison, USA)] and compared to a molecular size marker of a 100 bp (Fermentas, Thermo Scientific, USA). Amplified products were separated at 80 V for 1h30 and visualised under UV illumination (TFM-26 Ultra Transilluminator, UVP, Upland, CA). The images were captured using a digital gel documentation system (DigiDoc-It Imaging System, UVP, Upland)

5.2.5.3 Multiplex-PCR assay for the detection of genital mycoplasmas

All the specimens that were positive for the β-globin gene were subjected to a multiplex PCR (mPCR) assay to determine the presence of *M. genitalium*, *M. hominis*, *U. parvum* and *U. urealyticum*. The mPCR assay was adapted from the method by Stellrecht *et al*.[35]. The primers used in this assay targeted the 140-kDa adhesion protein of *M. genitalium*, the 16S rRNA of *M. hominis* and multiple-banded antigen (MBA) genes of *U. parvum* and *U. urealyticum* (Table 5.2). The mPCR assay was validated with the AmpliRun *Mycoplasma genitalium* DNA control (Vircell SL, Spain), a positive *M. hominis* sample isolated with A2 agar and reference strains ATCC 27813 (*U. parvum*) and ATCC 27619 (*U. urealyticum*).

The Qiagen multiplex PCR kit (Qiagen, Germany) was used according to the manufacturer's instructions (Table 5.3). Amplification was performed in a Gradient MasterCycler (Eppendorf, Hamburg, Germany) using the following 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 fragment size bands under UV illumination (TFM-26 Ultra Transilluminator, UVP, Upland, CA) after electrophoretic separation at 100 V for 1 h on a 2% (m/v) MetaPhor agarose gel (Lonza, USA) in 1X TBE buffer [45 mM Tris-borate (pH 8.0) (Sigma Chemical co, USA), 0.5 M EDTA (Promega, Madison, USA)]. A 50 kb ladder (Fermentas, Thermo Scientific, USA) was used to identify band sizes. Images were captured using a digital gel documentation system (DigiDoc-It Imaging System, UVP, Upland).

5.3 Statistical analysis

Quantitative data were expressed as percentages. Associations were determined using odds ratios (ORs), calculated with 2x2 contingency tables for different variables. A 95% confidence interval (CI) was considered significant (P-value <0.05).

5.4 Results

A total number of 220 pregnant women were included in the study. The low-risk group included 114 (51.8%) women, whereas 106 (48.2%) women were considered as high-risk based on obstetric history. The mean age of the population sampled for low-risk and high-risk were 29 years and 31 years, respectively. The median age of the population sampled for low-risk and high-risk women were 29 years and 30 years, respectively.

The mean and median gestational age of current pregnancies of participating women were 26 weeks. The breakdown of low-risk and high-risk women according to the three trimesters of gestational age is given in Table 5.4.

The low-risk factors identified are displayed in Table 5.5. Six women (5.3%) had no risk factor identified; 69 (60.5%) women had only one risk factor identified and 44 (38.6%) women had more than one risk factor identified. All participating women had a negative Rapid Plasma Reagin (RPR) status. Thirty-two women (28.1%) had risk factors identified ('other risk factors') not displayed in Table 5.5. These risk factors mainly included endometriosis, being rhesus negative, a bicornuate uterus and rheumatoid arthritis.

Table 5.6 represents the breakdown of the number of pregnancy losses in the high-risk group. Three women had a maximum number of seven pregnancy losses. Most women had between two and four pregnancy losses. The gestation period of pregnancy loss was available for 218 episodes. The total number of pregnancy loss is grouped in Table 5.7 according to trimester. The mean gestational age for pregnancy loss was 16 weeks, while the median was 8 weeks.

5.4.1 Culture

A total of 95 specimens were used to inoculate the Arginine and U9 broths. Only 51 specimens that were suspected of being positive (red colour) were sub-cultured from U9 broth onto solid A2 selective *Mycoplasma* agar medium. Many of the U9 broth cultures of these

specimens were turbid in colour, indicative of contamination. None of the Arginine broths were positive. After incubation of the A2 plates, two specimens (3.9%) were positive for *Ureaplasma* spp., four specimens (7.8%) were positive for *M. hominis*, three specimens (5.9%) were positive for *Ureaplasma* spp. and *M. hominis*, 28 specimens (54.9%) had contamination and 14 (27.5%) specimens were negative.

5.4.2 Microscopy

A total of 39 (17.7%) women tested positive for BV according to the Nugent scoring system. In the low-risk group, 22 (19%) women were BV positive, whereas 17 (16%) women were BV positive in the high-risk group (Table 5.8).

5.4.3 Multiplex PCR assay

All 220 specimens tested positive for the human β-globin gene (Figure 5.1). The results of the mPCR assay indicated that *U. parvum* was isolated from 157 specimens, whereas *U. urealyticum* was isolated from five specimens (Figure 5.2) (Table 5.9). One hundred specimens (87.7%) in the low-risk group and 85 specimens (80.2%) in the high-risk group had at least one genital mycoplasma species present.

Table 5.10 displays the breakdown of individual genital mycoplasma species that were positive in low-risk, high-risk and HIV-positive women. *Mycoplasma hominis* had p-values of p=0.0226 for low- and high-risk women and p=0.0020 for HIV-positive women.

5.4.4 Bacterial vaginosis and genital mycoplasmas according to Nugent score and gestational age of pregnancy

The positivity rate for specimens with at least one genital mycoplasma species present is 84% (185/220). Twenty-seven out of 33 (81.8%) specimens with an intermediate Nugent score had at least one genital mycoplasma species present (Table 5.11). Thirty-eight specimens out of 39 specimens (97.4%) with a high Nugent score (BV positive) were positive for at least one genital mycoplasma species. *Ureaplasma parvum* was present in 33 (84.6%) of BV positive specimens, whereas *M. hominis* was present in 28 (71.8%) of BV positive specimens (Table 5.12).

Bacterial vaginosis was isolated from eight women with first trimester pregnancies with an OR of 9.08 and a p-value of 0.0002 (Table 5.13). Twenty-one of 39 (53.8%) BV positive specimens and 4/5 (80%) of *U. urealyticum* positive specimens were isolated from women with second trimester pregnancies, whereas 19/33 (57.6%) of *M. genitalium*, 55/111 (49.5%) of *M. hominis* and 81/157 (51.6%) of *U. parvum* positive specimens were isolated from women with third trimester pregnancies (Table 5.13).

5.5 Discussion

In the present study, RTIs were highly prevalent in pregnant women. This implies that more women are at risk of having pregnancy complications, which will lead to an increased demand in health care assistance. The prevalence of BV found in the present study corresponds to the prevalence rates of 5% to 25% in pregnant women reported by other studies[36,37]. Bacterial vaginosis was not significantly associated with either low-risk (OR 1.25, 95% CI: 0.62 to 2.51, p=0.53) or high-risk (OR 0.80, 95% CI: 0.40 to 1.60, p=0.53) women. Tolosa and colleagues[36] studied the prevalence of BV in asymptomatic pregnant women in eight different institutions from different countries, including Colombia, Ireland, Myanmar, The Philippines, Thailand (2 institutions), The United States of America and Zimbabwe. The prevalence of BV in the present study ranks second highest, just after Zimbabwe, when comparing it to the prevalence rates found by Tolosa *et al*.[36]. However, the latter study reported the prevalence of BV in asymptomatic women only. In the present study, the investigator failed to discriminate between symptomatic and asymptomatic women with regards to BV. The symptoms that women reported were vague and subjective. Most of the women could not distinguish between a discharge characteristic of BV or a physiological discharge[38]. Some women described having a discharge, which may be more characteristic of and due to other vaginal infections, such as those caused by *Candida* spp. or *Trichomonas vaginalis*[38,39].

Genital mycoplasmas were poorly recovered from A2 agar medium and contamination was high (54.9% of specimens). Low recovery rates of genital mycoplasma isolation from clinical specimens have been recorded previously[40,41]. Mycoplasmas are difficult to culture and are susceptible to hostile environmental conditions, such as toxic metabolites and fluctuations in temperature[42]. Due to the high rate of contamination and financial limitations of this study, the cultivation of genital mycoplasmas with a culture method was discontinued.

The overall prevalence of genital mycoplasmas in the present study (as determined by PCR) was high (84%) when compared to other studies. Bayraktar and colleagues[43] reported a prevalence of 29% for genital mycoplasmas in Turkey, while Koh *et al.*[44] found a prevalence of 44.2% in Korea. *Ureaplasma parvum* was the most detected species and contributed to the high prevalence of genital mycoplasmas found in the present study. Similarly, Kacerovsky and co-workers[45] found *U. parvum* to be the most prevalent genital mycoplasma in sexually active women. Some studies, including a study from South Africa, have reported low (6% to 15%) prevalence rates of *U. urealyticum*[45,46], comparable to the prevalence found in the present study, whereas other studies reported high (24% to 63%) prevalence rates[44,47]. Although *U. urealyticum* has been implicated in more pathogenic cases[48,49], inconsistent findings exist as to which *Ureaplasma* spp. is the most pathogenic[23,50]. *Mycoplasma genitalium* and *M. hominis* were more prevalent in the present study compared to other studies[46,47,51].

Mycoplasma hominis is the only species that was associated significantly more with low-risk women (p=0.023) than with high-risk women. This species was the only genital mycoplasma that was significantly isolated from HIV-positive women (OR 3.57, 95% CI: 1.59 to 8.01, p=0.002). This contradicts the finding of another South African study by Govender *et al.* (2010) who reported no association between the colonisation of *M. hominis* and HIV status.

Mycoplasma hominis is reported to be present in 58% to 76% in women with BV and *U. urealyticum* in 62% to 92% of women with BV[52]. The findings of *M. hominis* in the present study is similar to what Hill[52] reported as this species was present in 71.7% of women with BV. *Ureaplasma parvum* was the species that was present in a high number (84.6%) of women with BV. Genital mycoplasmas were significantly associated with BV when at least one mycoplasma sp. was considered (OR 8.79, 95% CI: 1.17 to 66.28, p=0.035). Keane *et al.*[5] studied the four genital mycoplasma species as possible causes for BV and detected *M. hominis* significantly (p=0.0001) more in women with BV than in those without BV. Zariffard and colleagues[53] reported similar results. When individual *Mycoplasma* spp. were considered in the present study, *M. hominis* (OR 3.01, 95% CI: 1.41 to 6.40, p=0.004) and *U. parvum* (OR 2.53, 95% CI: 1.003 to 6.37, p=0.049) were significantly isolated from women with BV.

Bacterial vaginosis was significantly isolated from women with first trimester pregnancies (95% CI: 2.789 to 29.586; p=0.0002). The magnitude of BV measured in the first trimester of pregnancy may lead to an elevated risk of second trimester pregnancy loss[54]. Genital mycoplasmas were isolated more in the second and third trimesters; however, these findings were not statistically significant. All the individual mycoplasma species, except *U. urealyticum*, was present in higher numbers in specimens with a high Nugent score than in specimens with an intermediate Nugent score.

The present study established an association between BV and *M. hominis* and *U. parvum*. The Nugent scoring system lacks sensitivity as it does not allow the identification of genital mycoplasmas and *A. vaginae*, bacterial species that are often implicated in BV[55,56]. In the present study, selective culture methods fail to successfully recover genital mycoplasmas. This may lead to the misidentification and underdetection of RTIs, such as BV and genital mycoplasmas[27]. The association of these bacteria with poor pregnancy outcomes highlights the need for more accurate identification methods.

There was no difference in the isolation of BV and genital mycoplasmas from low-risk and high-risk women, except for *M. hominis*, which was more associated with low-risk women. The prevalence of these bacterial species in both study groups were high compared to similar studies. This may be an indication that both groups of women are equally susceptible to infection and equally at risk for poor obstetric outcomes due to infection.

It is acknowledged that this study had limitations. Women in the high-risk group were women who had a history of at least one pregnancy loss, irrespective of the date of the loss. It would therefore not be possible to say whether current colonisation by BV associated bacteria, including genital mycoplasmas, contributed to the loss or increased women's susceptibility to a pregnancy loss. These findings can be investigated by only including women with recent adverse pregnancy outcomes or by conducting an outcome-based study within two different time frames where women are followed up. This could not be done in the present study due to limited time. The widely reported association between BV and HIV[57,58,59] could not be established in this study. Even though a similar observation was made by Demba *et al.*[60], it is recommended to compare the prevalence of BV and genital mycoplasmas in an HIV-positive population to obtain more clinically significant results.

5.6 Conclusion

In this study, genital mycoplasmas were found to be significantly associated with BV. The species that were associated with BV were *M. hominis* and *U. parvum*. Several of these infections are asymptomatic and may not be detected. The fact that *M. hominis* is associated with HIV poses health risks for the mother and foetus. The potential impacts of BV associated genital mycoplasmas that are highly prevalent should be investigated in more defined populations, focusing on maternal and foetal health. Multiplex PCR assays would be of more value to conduct such investigations as these assays are more sensitive than conventional culture methods that are more prone to contamination.

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TABLES AND FIGURES

Table 5.1: Oligonucleotide sequences of primers used in the singleplex PCR to amplify the human β-globin gene (Martin *et al.*, 2009)

Target	DNA sequence (5'-3')*	Expected size (bp)
Human β-globin gene	Forward primer: -CAAGGTGAACGTGGATGAAG- Reverse primer: -CCTGAAGTTCTCAGGATCCACG-	395 bp

*Primers synthesised by Inqaba Biotechnical Industries, Pretoria, South Africa

Table 5.2: Targets and oligonucleotide sequences of primers used in the mPCR assay for the detection of genital mycoplasmas (Stellrecht *et al.*, 2004)

Mycoplasma species	Target	Primer name and DNA sequence (5'-3')*	Expected sizes (bp)
M. genitalium	140-kDa adhesion protein	MG1: -AGTTGATGAAACCTTAACCCCTTGG- MG2: -CCGTTGAGGGGTTTTCCATTTTTGC-	282 bp
M. hominis	16S rRNA	RNAH1: -CAATGGCTAATGCCGGATACGC- RNAH2: -GGTACCGTCAGTCTGCAAT-	334 bp
U. urealyticum* and *U. parvum	MBA gene	UMS125: -GTATTTGCAATCTTTATATGTTTTCG- UMA226: -CAGCTGATGTAAGTGCAGCATTAAA TTC-	403 or 448 bp

*Primers synthesised by Inqaba Biotechnical Industries, Pretoria, South Africa

Table 5.3: Reaction setup and components used in the mPCR assay for the detection of genital mycoplasmas

Component	Volume/ reaction	Final concentration
Reaction mixture		
▪ **2X Qiagen Multiplex PCR Master Mix**	12.5 µl	1x
▪ **10X primer mix, 2 µM each primer**	2.5 µl	0.2 µM
▪ **Q-solution**	2.5 µl	-
▪ **RNase-free water**	4.5 µl	
Template DNA	3 µl	≤ 1 µg DNA/50 µl
Total volume	**25 µl**	

Table 5.4: The number of low- and high-risk women according to trimester of pregnancy

	LR	HR	Total
1st trimester (0 to 12 weeks)	6	7	13
2nd trimester (13 to 26 weeks)	49	49	98
3rd trimester (27 to 40 weeks)	59	50	109
Total	**114**	**106**	**220**

Table 5.5: Risk factors identified in the low-risk group (n=114) and the number of women with the specified condition

	Number of women (%)
Advanced maternal age (AMA)	25 (21.9%)
Asthma	4 (3.5%)
Cardiac	18 (15.7%)
Deep vein thrombosis (DVT)	3 (2.6%)
Diabetes	20 (17.5%)
Epilepsy	7 (6.1%)
Fibroids	6 (5.2%)
Hypertension	19 (16.7%)
Kidney-related conditions	6 (5.2%)
Pre-eclampsia toxaemia (PET)	4 (3.5%)
Previous caesarean section	16 (14%)
Thyroid-related conditions	7 (6.1%)
Twin pregnancy	12 (10.5%)
Other risk factors	32 (28.1%)

Table 5.6: The breakdown of the number of pregnancy losses in high-risk women

	1 pregnancy loss No (%)	2 to 4 pregnancy losses No (%)	>4 pregnancy losses No (%)	Total
Number of women	45 (42.5)	56 (52.8)	5 (4.7)	106

Table 5.7: The gestational age of pregnancy losses in high-risk women according to trimester

	1st trimester No (%)	2nd trimester No (%)	3rd trimester No (%)
Number of losses (n=218)	111 (51%)	79 (36.2%)	28 (12.8%)

Table 5.8: Bacterial vaginosis diagnosis according to Nugent score

	Negative (0 to 3)	Intermediate (4 to 6)	Positive (7 to 10)	OR	95% CI	P-value
LR (n=114)	75	17	22	1.25	0.623 to 2.513	0.5274
HR (n=106)	73	16	17	0.80	0.398 to 1.603	0.5274
HIV + (n=36)	18	9	9	1.71	0.732 to 4.003	0.2154

Table 5.9: The number of low- and high-risk women that were positive for each genital mycoplasma species

	LR (n=114) No. (%)	HR (n=106) No. (%)	Total (n=220) No. (%)
M. genitalium	17 (14.9)	16 (15.1)	33 (15)
M. hominis	66 (57.9)	45 (42.5)	111 (50.5)
U. parvum	87 (76.3)	70 (66.3)	157 (71.4)
U. urealyticum	2 (1.75)	3 (2.8)	5 (2.3)

Table 5.10: The association of different genital mycoplasma species with low- and high-risk and HIV positive women

		Positive	Negative	OR	95% CI	P-value
	LR	17	97	0.99	0.470 to 2.067	0.9699
M. genitalium	**HR**	16	90	1.01	0.484 to 2.127	0.9699
	HIV +	8	28	1.82	0.745 to 4.433	0.1893
	LR	66	48	1.86	1.091 to 3.184	0.0226
M. hominis	**HR**	45	61	0.54	0.314 to 0.916	0.0226
	HIV +	27	9	3.57	1.592 to 8.014	0.0020
	LR	87	27	1.66	0.919 to 2.989	0.0933
U. parvum	**HR**	70	36	0.60	0.335 to 1.089	0.0933
	HIV +	26	10	1.05	0.475 to 2.332	0.9008
	LR	2	112	0.61	0.100 to 3.743	0.5961
U. urealyticum	**HR**	3	103	1.63	0.267 to 9.958	0.5961
	HIV +	2	34	3.55	0.571 to 22.043	0.1740

Table 5.11: The number of BV-positive, -intermediate and -negative specimens with at least one mycoplasma species present

	No. of specimens with at least one mycoplasma sp. present	OR	95% CI	P-value
Negative (n=148)	120	0.46	0.191 to 1.114	0.0856
Intermediate (n=33)	27	0.83	0.313 to 2.177	0.6990
Positive (n=39)	38	8.79	1.166 to 66.278	0.0350

Table 5.12: Association of various genital mycoplasma species with BV

	Negative	Intermediate	Positive	OR	95% CI	P-value
M. genitalium **(n=33)**	21	5	7	1.30	0.521 to 3.263	0.5705
M. hominis **(n=111)**	65	18	28	3.01	1.411 to 6.403	0.0043
U. parvum **(n=157)**	102	22	33	2.53	1.003 to 6.374	0.0493
U. urealyticum **(n=5)**	3	1	1	1.16	0.127 to 10.713	0.8930

Table 5.13: The association of BV and the different genital mycoplasma species with the three different trimesters of pregnancy

		Positive	OR	95% CI	P-value
BV	**1st trimester**	8	9.08	2.789 to 29.586	0.0002
	2nd trimester	21	1.58	0.786 to 3.158	0.1998
	3rd trimester	10	0.29	0.131 to 0.621	0.0016
M. genitalium	**1st trimester**	1	0.46	0.057 to 3.628	0.4578
	2nd trimester	13	0.78	0.367 to 1.660	0.5191
	3rd trimester	19	1.46	0.693 to 3.089	0.3188
M. hominis	**1st trimester**	5	0.60	0.189 to 1.881	0.377
	2nd trimester	51	1.12	0.659 to 1.909	0.673
	3rd trimester	55	1.00	0.590 to 1.697	0.999
U. parvum	**1st trimester**	9	0.90	0.266 to 3.026	0.8608
	2nd trimester	67	0.77	0.428 to 1.382	0.3788
	3rd trimester	81	1.33	0.741 to 2.397	0.3384
U. urealyticum	**1st trimester**	0	-	-	-
	2nd trimester	4	4.87	0.535 to 44.257	0.1601
	3rd trimester	1	0.32	0.0345 to 2.872	0.3062

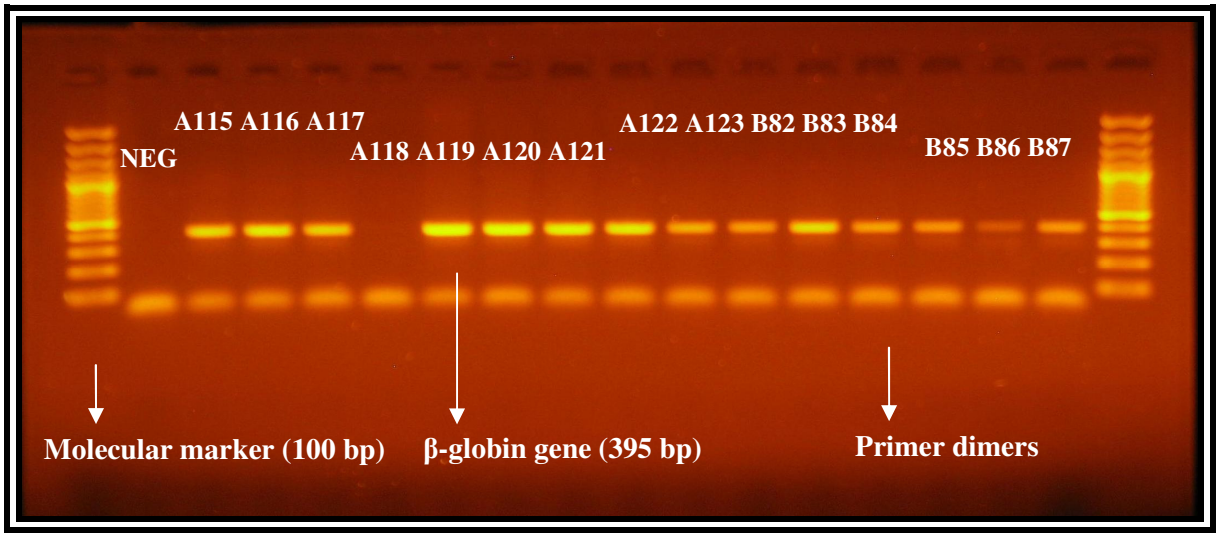

Figure 5.1: **Gel electrophoresis analysis of a singleplex PCR assay for the detection of the human β-globin gene on a 1% (m/v) agarose gel (Lonza, USA). Bands for specimens A115 to A123 and specimens B82 to B87 are displayed with the exception of specimen A118, which was positive in a subsequent singleplex PCR run**

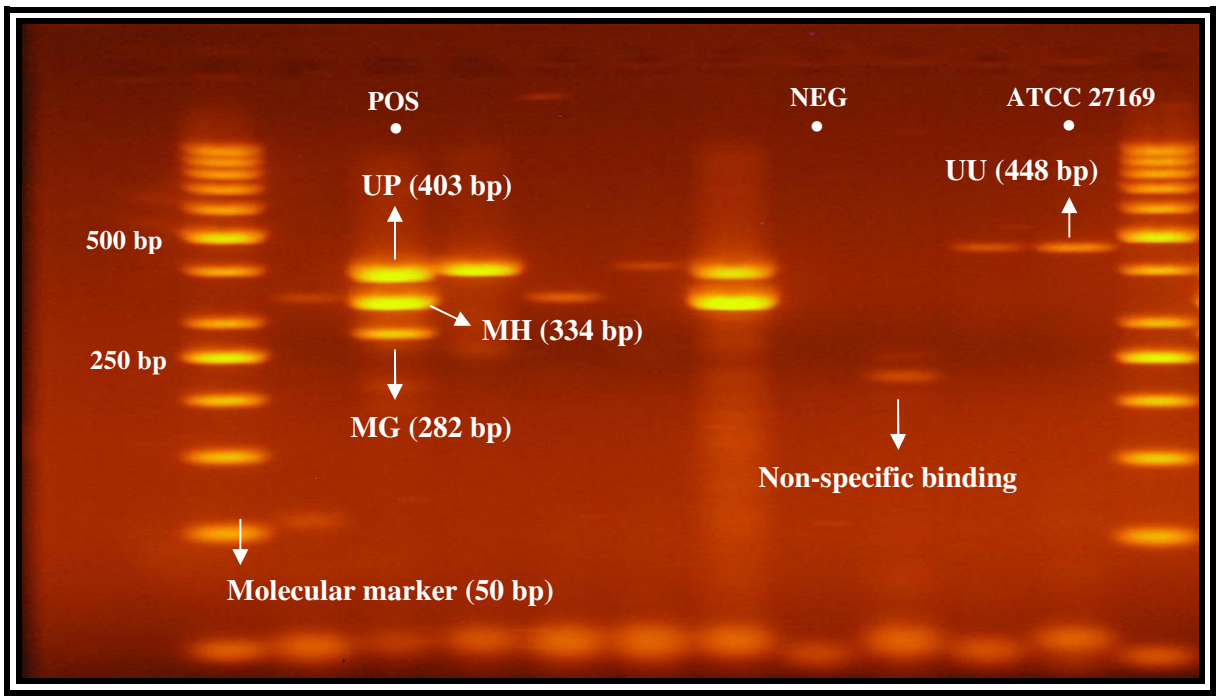

Figure 5.2: **Gel electrophoresis analysis of an mPCR assay for the detection of genital mycoplasmas on a 2% (m/v) MetaPhor agarose gel (Lonza, USA). Bands are displayed for a mixture of positives (containing *M. genitalium* DNA control, A2-isolated *M. hominis* and *U. parvum* ATCC27813) and *U. urealyticum* ATCC27619**

CHAPTER 6

The quantification of *Atopobium vaginae* and *Gardnerella vaginalis* in vaginal specimens

Abstract

Background Bacterial vaginosis is a reproductive tract infection, which is the most common cause of vaginal discharge in women of reproductive age. *Atopobium vaginae* and *Gardnerella vaginalis* are the bacterial species most frequently isolated in positive cases and may play major roles in the pathogenesis of BV. The purpose of this study was to quantify *A. vaginae* and *G. vaginalis* in vaginal specimens and determine threshold values to predict BV. **Methods** Pregnant women with (high-risk) and without (low-risk) a history of at least one pregnancy loss were included in the study. Self-collected vaginal swabs were obtained to diagnose BV with the Nugent scoring system and subsequently quantify *A. vaginae* and *G. vaginalis* with a real-time PCR assay. **Results** Thirty-nine women (17.7%) were positive for BV, whereas 33 (15%) of the specimens had intermediate Nugent scores. The threshold concentrations of *A. vaginae* and *G. vaginalis* that were significantly associated with high Nugent scores were 10^6 to 10^7 copies/reaction. **Conclusions** Concentrations of 10^6 to 10^7 copies/reaction of *A. vaginae* and *G. vaginalis* can be used to predict BV in women. The Nugent scoring system lacks sensitivity due to its inability to distinctly detect *A. vaginae*. Quantitative PCR assays prove promising to help elucidate the roles and pathogenesis of different bacterial species involved in BV.

Keywords: *Atopobium vaginae*, Bacterial vaginosis, *Gardnerella vaginalis*, Nugent score, Quantification, qPCR assay

6.1 Introduction

Bacterial vaginosis (BV) is the most common reproductive tract infection (RTI) of women of reproductive age (Marconi *et al*., 2012). This condition is characterised by the depletion of the dominant *Lactobacillus* flora and the overgrowth of anaerobic and facultative bacteria (O'Hanlon *et al*., 2011). A BV dominated flora have been reported to increase the risk for sexually transmitted infection (STI) acquisition in women, including the human immunodeficiency virus (HIV) (Myer *et al*., 2005; Lamont *et al*., 2011). During pregnancy, BV may reach prevalence rates of up to 55% and is associated with a two-fold risk of preterm delivery (Hogan *et al*., 2007; Menard *et al*., 2010).

Even though BV is reported to be a polymicrobial condition (Patterson *et al*., 2010), evidence suggests that there is an inverse relationship between the disappearance of *Lactobacillus* spp. and an increase in the concentrations of *Atopobium vaginae* and *Gardnerella vaginalis* in particular (Menard *et al*., 2008; Menard *et al*., 2010). Menard *et al*. (2008) isolated *A. vaginae*, together with *G. vaginalis*, from BV positive specimens in higher concentrations than other bacterial species. These findings are supported by similar studies (Bradshaw *et al*., 2006; Fredricks *et al*., 2007; Trama *et al*., 2008).

A study by Swidsinski *et al*. (2010) found that *G. vaginalis* are present in two forms, namely planktonic (dispersed) and biofilm-associated (cohesive). The mode of growth that is followed is thought to be strain-dependent (Verstraelen and Swidsinski, 2013). It is presumed that *G. vaginalis* is the first bacterial species to adhere to the vaginal epithelium and subsequently becomes a scaffolding to which other bacterial species adhere (Verstraelen and Swidsinski, 2013). Nonetheless, the lack of a proper *in vitro* model of the BV biofilm and the poor understanding of the association between *A. vaginae* and *G. vaginalis* still complicate the pathogenesis of BV (Verstraelen and Swidsinski, 2013).

The Nugent scoring system is one of the gold standard methods of detecting BV, the other being Amsel's criteria (Danielsson *et al*., 2011). *Atopobium vaginae* is not readily detected by the Nugent scoring system (Brotman and Ravel, 2008). Menard and co-workers (2008) proposed the use of a quantitative real-time PCR (qPCR) assay, which quantifies the levels of DNA in vaginal specimens to predict BV. The researchers reported a reproducible quantitative tool that uses cut-off values for the concentrations of *A. vaginae* and *G. vaginalis*

to establish the molecular diagnosis of BV (Menard *et al*., 2008). The molecular criteria to predict BV had positive and negative predictive values of 96% and 99%, respectively (Menard *et al*., 2008).

The purpose of this study was to quantify *A. vaginae* and *G. vaginalis* in vaginal specimens. The Nugent score was used as a reference to determine *A. vaginae* and *G. vaginalis* concentrations to predict BV with quantitative PCR (qPCR).

6.2 Materials and Methods

Two self-collected vaginal swabs (Copan Diagnostics, Inc., Italy) were obtained from pregnant women attending the antenatal and Maternal and Foetal Unit (MAFU) clinics of a tertiary academic hospital in Pretoria, Gauteng, South Africa. A dry Rayon swab was used to prepare vaginal smears on glass slides (B&C, Germany) immediately after the swab was taken and left to air dry. An eSwab (a flocked nylon swab) was used to collect vaginal specimens and subsequently seed 1 ml of modified Amies transport medium (Copan Diagnostics, Inc., Italy). The order in which the dry swab and eSwab were obtained was alternated between patients. Vaginal specimens were stored on ice and transported to the laboratory within 1 h to 4 h of collection.

6.2.1 Microscopy

Upon arrival at the laboratory, smears were fixed to slides by heating for 5 s using a Bunsen burner, Gram-stained and graded with the Nugent scoring system as described by Nugent *et al*. (1991). On a scale from zero to ten, a score of 0 to 3 was considered negative for BV, a score of 4 to 6 was considered intermediate and a score of 7 to 10 was considered positive for BV (Appendix B). As a quality control measure, all the slides were re-read at a later stage and were also graded by a second examiner. Some slides were also compared to previously graded slides that served as controls to confirm the Nugent score.

6.2.2 DNA extraction from modified Amies transport medium

Bacterial DNA was isolated from the vaginal swabs with the ZR Fungal/Bacterial DNA kit (Zymo Research, USA) according to the manufacturer's instructions (Appendix B). A volume of 150 µl seeded Amies transport medium (Copan Diagnostics, Inc, Italy) was used to extract

bacterial DNA. One hundred microliters of ultra-pure DNA was eluted and stored at -20ºC until further analysis.

6.2.3 Quantification of *A. vaginae* and *G. vaginalis* positive controls

The initial concentrations of the *A. vaginae* and *G. vaginalis* DNA positive controls were unknown. The initial concentrations were determined by subjecting each positive control to a conventional singleplex PCR in triplicate, followed by a DNA purification step. This was done in order to obtain as pure and specific *A. vaginae* and *G. vaginalis* DNA as possible, free of other extracted bacterial or fungal DNA.

6.2.3.1 Singleplex PCR assay

The Qiagen multiplex PCR kit (Qiagen, Germany) was used in the singleplex PCR assay. A final reaction volume of 25 µl was used and comprised 12.5 µl of 2X Qiagen Multiplex PCR Master Mix, 2.5 µl of 0.4 µM forward and reverse primer (Table 6.1) (Menard *et al*., 2008), 7 µl RNase-free water (Qiagen, Germany) and 3 µl of template DNA.

Amplification was performed in a Gradient MasterCycler (Eppendorf, Hamburg, Germany). The PCR protocol consisted of initial denaturation step of 95ºC for 15 min, 45 cycles of two steps including denaturation at 94°C for 60 s, combined annealing and extension at 60°C for 60 s, followed by a final extension step at 72°C for 7 min. Amplified products were visualised for specific fragment size bands under UV illumination (TFM-26 Ultra Transilluminator, UVP, Upland, CA) after electrophoretic separation at 100 V for 1 h on a 2% (m/v) MetaPhor agarose gel (Lonza, USA) in 1X TBE buffer [45 mM Tris-borate (pH 8.0) (Sigma Chemical co, USA), 1 mM EDTA (Promega, Madison, USA)]. A 50 kb ladder (Fermentas, Thermo Scientific, USA) was used to identify band sizes. The product size for *A. vaginae* was 88 bp and for *G. vaginalis* 92 bp (Menard *et al*., 2008). Images were captured using a digital gel documentation system (DigiDoc-It Imaging System, UVP, Upland). The amplicons were purified directly after PCR instead of excising the products from the gel to minimise contamination.

6.2.3.2 DNA purification [Zymoclean Gel DNA Recovery Kit (Zymo Research, USA)]

Initially, 24 ml 100% ethanol (Merck, Germany) was added to every 6 ml DNA Wash Buffer (Zymo Research, USA) concentrate to obtain the final DNA Wash Buffer solution. Three volumes (75 µl) of ABD solution (Zymo Research, USA) were added to each volume (25 µl) of amplified product. The samples were incubated at 55°C for 10 min. The solution was transferred to a Zymo-Spin I Column (Zymo Research, USA) in a Collection Tube (Zymo Research, USA) and centrifuged (Spectrafuge centrifuge; Labnet international, USA) at 10 000 *x g* for 30 s. The flow-through was discarded. Wash Buffer (200 µl) (Zymo Research, USA) was added to the column, which was centrifuged (Spectrafuge centrifuge; Labnet international, USA) at 10 000 *x g* for 30 s and the flow-through was discarded. The wash step was repeated. Thirty microliters of water was added directly to the column matrix, which was transferred to a 1.5 ml Eppendorf tube (Lasec, South Africa) and centrifuged (Spectrafuge centrifuge; Labnet international, USA) at 10 000 *x g* for 30 s to elute the DNA.

6.2.3.3 Concentration determination

The purified DNA of *A. vaginae* and *G. vaginalis* were serially diluted (1:10). This was done for each sample in triplicate up to dilution 10^{-4}. The concentrations (ng/µl) of the samples were determined with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, USA).

The undiluted amplicons were used to calculate the number of copies per reaction. It was calculated with the formula (URI Genomics and Sequence centre, 2004):

$$\frac{\textbf{(Concentration of DNA in ng/µl x } 10^{-9}\textbf{)}}{\textbf{(Number of nucleotides x mass of a nucleotide)}} \times \frac{\textbf{Avogadro's constant}}{\textbf{(6.022 x } 10^{23}\textbf{ g/mole)}}$$

and is based on the assumption that the average weight of a DNA nucleotide is 340 Daltons

The positive control for *A. vaginae* had a spectrophotometer reading of 2.69 ng/µl. The number of copies per reaction was calculated as follow:

$$[(2.69 \times 10^{-9})/ (176 \times 340)] \times (6.022 \times 10^{23})$$
$$= 2.7 \times 10^{10} \text{ copies/µl} \times 2.5 \text{ (µl per reaction)}$$

= 6.77E+10 copies per reaction

(6.77E+10 copies per reaction was diluted 1:10 to obtain 6.77E+09 copies per reaction, which was used as the initial concentration to generate a standard curve for *A. vaginae*)

The positive control for *G. vaginalis* had a spectrophotometer reading of 3.47 ng/µl. The number of copies per reaction was calculated as follow:

$$[(3.47 \times 10^{-9})/ (176 \times 340)] \times (6.022 \times 10^{23})$$

= 3.34 copies/µl x 2.5 (µl per reaction)

= 8.35E+10 copies per reaction

(8.35E+10 copies per reaction was used as the initial concentration to generate a standard curve for *G. vaginalis*)

6.2.4 Quantitative duplex real-time PCR assay for the generation of standard curves and quantification of *A. vaginae* and *G. vaginalis* DNA in vaginal specimens

Real-time PCR reactions were performed with TaqMan probes using a LightCycler® 480 (Roche Diagnostics, Germany). The TaqMan probe for *A. vaginae* was labelled with a carboxy-2', 4, 4', 5', 7, 7'-hexachlorofluoroscein (HEX) reporter dye at the 5' end and a black hole quencher (BHQ-1) at the 3' end, whereas the probe for *G. vaginalis* was labelled with a 6-carboxyfluorescein (6-FAM) reporter dye at the 5' end and a BHQ-1 quencher at the 3' end. The DNA targets as well as the primers and probe sequences for *G. vaginalis* and *A. vaginae* are displayed in Table 6.1.

The QuantiTect Multiplex PCR NoROX kit (Qiagen, Germany) was used to perform the real-time qPCR reactions (Table 6.2). The amplification programme was run at 95°C for 15 min, followed by 45 two-step cycles at 94°C for 60 s (denaturation) and at 60°C for 1 min (combined annealing and extension) (Menard *et al*., 2008). Fluorescence of the products was measured in real-time after extension of the annealed primers by using the HEX channel for *A. vaginae* and FAM channel for *G. vaginalis*. The HEX channel absorbs fluorescence at wavelengths of 533 nm to 580 nm and the FAM channel at 465 nm to 510 nm (Roche Diagnostics, Germany).

The positive controls for the qPCR assay consisted of DNA extracted from a positive *A. vaginae* isolate and *G. vaginalis* ATCC strain 14018. A dilution series of the *A. vaginae* (initial concentration of 6.77E+09 copies/reaction) and *G. vaginalis* (initial concentration of 8.35E+10 copies/reaction) positive controls were made. Singleplex PCRs were performed in triplicate for every dilution of each positive control. The products of the singleplex PCRs were used to generate separate standard curves for *A. vaginae* and *G. vaginalis* and were stored in the database (Figures 6.1 to 6.4).

The unknown specimens were subjected to duplex qPCRs that were performed in dual fluorescence. Quantification of *A. vaginae* and *G. vaginalis* in each specimen was done by comparing the amplified products to the respective standard curves to obtain extrapolated concentrations for both *A. vaginae* and *G. vaginalis*.

6.3 Results

A total of 220 vaginal specimens were collected. One hundred and forty-eight (67.3%) specimens were negative for BV (Nugent score 0 to 3), 33 (15%) specimens had an intermediate Nugent score (4 to 6) and 39 (17.7%) specimens were BV positive (Nugent score 7 to 10).

The mean concentrations of *A. vaginae* in low- and high-risk women were between 10^5 to 10^6 copies/reaction. The median concentration of *A. vaginae* in low-risk women was between 10^2 to 10^3 copies/reaction and in high-risk women between 10^1 to 10^2 copies/reaction. *Atopobium vaginae* was detected at concentrations ranging between 10^1 to 10^2 copies/reaction when all specimens were considered (Table 6.3). Sixteen out of 39 (41%) BV positive specimens had *A. vaginae* concentrations between 10^6 to 10^7 copies/reaction (Figure 6.5).

The mean concentrations of *G. vaginalis* in low- and high-risk women were between 10^5 to 10^6 copies/reaction. The median concentration of *G. vaginalis* in low-risk women was between 10^2 to 10^3 copies/reaction and in high-risk women between 10^1 to 10^2 copies/reaction. Seventy-one out of 220 (32.3%) specimens were negative for *G. vaginalis* (Table 6.4). Fifteen out of 39 (38.5%) BV positive specimens had *G. vaginalis* concentrations between 10^5 to 10^6 copies/reaction (Figure 6.6).

6.4 Discussion

The results of this study indicated that *A. vaginae* and *G. vaginalis* were significantly present in high concentrations in BV positive specimens. Specimens with Nugent scores 9 and 10 had *A. vaginae* present at concentrations of $>10^4$ copies/reaction (Figure 6.5). Similarly, the scatter plot displays that most specimens with intermediate Nugent scores had *A. vaginae* at concentrations of $>10^4$ copies/reaction. Most specimens with a Nugent score of zero had *A. vaginae* concentrations $<10^4$ copies/reaction. Similar to *A. vaginae*, specimens with Nugent scores 9 and 10 all had *G. vaginalis* at concentrations of $>10^3$ copies/reaction (Figure 6.6). The number of specimens with an intermediate Nugent score and *G. vaginalis* at concentrations of $>10^4$ copies/reaction was higher than the number of specimens with an intermediate Nugent score and *A. vaginae* concentrations $>10^4$ copies/reaction.

The quantification of *A. vaginae* and *G. vaginalis* by Menard *et al*. (2008) was more specific to predict BV than the mere presence of these bacteria. These researchers found that the combination of threshold *A. vaginae* DNA levels of $\geq 10^8$ copies/ml and threshold *G. vaginalis* DNA levels of $\geq 10^9$ copies/ml was the best diagnostic definition of BV (Menard *et al*., 2008). In the present study, *A. vaginae* concentrations of 10^6 to 10^7 copies/reaction (10^8 to 10^9 copies/ml) were determined as the threshold level as these concentrations had the highest OR that was statistically significant (OR 10.75; 95% CI: 4.448 to 25.987; $p<0.0001$) (Zozaya-Hinchliffe *et al*., 2010). *Gardnerella vaginalis* concentrations of 10^6 to 10^7 copies/reaction (10^8 to 10^9 copies/ml) were determined as the threshold level (OR 10.56; 95% CI: 3.311 to 33.678; $p=0.0001$). The elevated concentrations of *A. vaginae* and *G. vaginalis* observed in women who are positive for BV are similar to what other studies reported (Menard *et al*., 2008; Zozaya-Hinchliffe *et al*., 2010). These threshold values may be used as cut-off values to predict BV. It is important to note that *A. vaginae* was detected at $<10^4$ copies/reaction in 4/39 BV cases (± 10%) and *G. vaginalis* was detected at $<10^4$ copies/reaction in 8/39 BV cases (± 20%). This could be the result of BV-associated bacteria that are present in low copy numbers but still gave a positive result with the Nugent scoring system. Nonetheless, the use of threshold values first need to be prospectively applied to vaginal specimens to determine the accuracy in predicting BV. Figures 6.5 and 6.6 demonstrated that *A. vaginae* and *G. vaginalis* were present in a large proportion of women who had normal Nugent scores. This is similar to the findings of Zozaya-Hinchliffe *et al*. (2010) who studied young women

without a recent history of antimicrobial agent use presenting for routine STD assessment in New Orleans, USA.

Fifteen percent (33/220) of all specimens were graded as intermediate, which corresponds to the 8% to 22% reported in the literature (Larsson *et al*., 2004; Guerra *et al*., 2006; Libman *et al*., 2006). This category with its mixed bacterial flora is largely uncharacterised and complicates clinical approaches (Libman *et al*., 2006; Menard *et al*., 2010). Intermediate flora is generally not treated as it may be a transitional state between normal flora and BV or *vice versa* (Verhelst *et al*., 2005; Ugwumadu, 2007). However, intermediate flora is equally associated with poor obstetric outcomes and PCR assays propose a profile more similar to that of BV (Bradshaw *et al*., 2006; Ugwumadu, 2007). The findings of this study support this statement as most of the specimens with intermediate flora had increased concentrations of *A. vaginae* and *G. vaginalis* (Figures 6.5 and 6.6).

6.5 Conclusion

Atopobium vaginae and *G. vaginalis* concentrations of 10^6 to 10^7 copies/reaction (10^8 to 10^9 copies/ml) may be used as cut-off values to predict BV. The similar profiles of vaginal specimens with intermediate and high Nugent scores highlight the need for more defining and inclusive diagnostic assays. Continued research is needed to determine the roles of the different bacterial species in the pathogenesis of BV and qPCR assays are reproducible diagnostic assays that may assist in such investigations.

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TABLES AND FIGURES

Table 6.1: Targets and oligonucleotide sequences of primers and probes used for the quantification of genes specific for *G. vaginalis* and *A. vaginae* (Menard *et al*., 2008)

Bacterial species	DNA target	Oligonucleotide sequences of primers and probes (5'-3')*
A. vaginae	16S rRNA	Forward: -CCCTATCCGCTCCTGATACC- Reverse: -CCAAATATCTGCGCATTTCA- Probe: HEX-GCAGGCTTGAGTCTGGTAGGGGA-BHQ-1
G. vaginalis	*Cpn* 60 (Chaperonin 60)	Forward: -CGCATCTGCTAAGGATGTTG- Reverse: -CAGCAATCTTTTCGCCAACT- Probe: FAM-TGCAACTATTTCTGCAGCAGATCC-BHQ-1

*All primers and probes were synthesised by Inqaba Biotechnical Industries, Pretoria, South Africa

Table 6.2: Reaction setup and components for the QuantiTect Multiplex PCR assay for the detection and quantification of *G. vaginalis* and *A. vaginae* (Qiagen, Germany)

Component	Volume/reaction	Final concentration
Reaction mixture		
▪ **2X QuantiTect Multiplex PCR NoROX Master Mix**	10 µl	
▪ **20X primer–probe mix 1 (*A. vaginae*)**	1 µl	0.4 µM forward primer 1; 0.4 µM reverse primer 1; 0.2 µM probe 1
▪ **20X primer–probe mix 2 (*G. vaginalis*)**	1 µl	0.4 µM forward primer 2; 0.4 µM reverse primer 2; 0.2 µM probe 2
▪ **RNase-free water**	15 µl	-
Template DNA	5 µl	≤ 500 ng/reaction
Total volume	**20 µl**	

Table 6.3: The distribution of *A. vaginae* at different concentrations in all specimens and in BV positive specimens

DNA Concentration (copies/reaction)	All specimens (n = 220)	BV positive specimens (n=39)	OR (*A. vaginae* and BV positive specimens)	95% CI	P-value
Negative	45	2	0.17	0.040 to 0.750	0.0190
< 10	23	0	-	-	-
10^1 to 10^2	50	1	0.07	0.010 to 0.530	0.0100
10^2 to 10^3	16	0	-	-	-
10^3 to 10^4	12	1	0.41	0.051 to 3.246	0.3959
10^4 to 10^5	24	11	5.08	2.070 to 12.451	0.0004
10^5 to 10^6	21	7	2.61	0.976 to 6.973	0.0558
10^6 to 10^7	27	16	10.75	4.448 to 25.987	<0.0001
10^7 to 10^8	2	1	4.74	0.290 to 77.419	0.2752

Table 6.4: The distribution of *G. vaginalis* at different concentrations in all specimens and in BV positive specimens

DNA Concentration (copies/reaction)	All specimens (n = 220)	BV positive specimens (n=39)	OR (*G. vaginalis* and BV positive specimens)	95% CI	P-value
Negative	71	3	0.14	0.041 to 0.467	0.0014
< 10	10	2	1.17	0.239 to 5.730	0.8474
10^1 to 10^2	31	1	0.13	0.018 to 1.002	0.0503
10^2 to 10^3	16	0	-	-	-
10^3 to 10^4	17	2	0.60	0.131 to 2.729	0.5070
10^4 to 10^5	26	7	1.87	0.724 to 4.803	0.1965
10^5 to 10^6	35	15	5.03	2.272 to 11.142	0.0001
10^6 to 10^7	14	9	10.56	3.311 to 33.678	0.0001

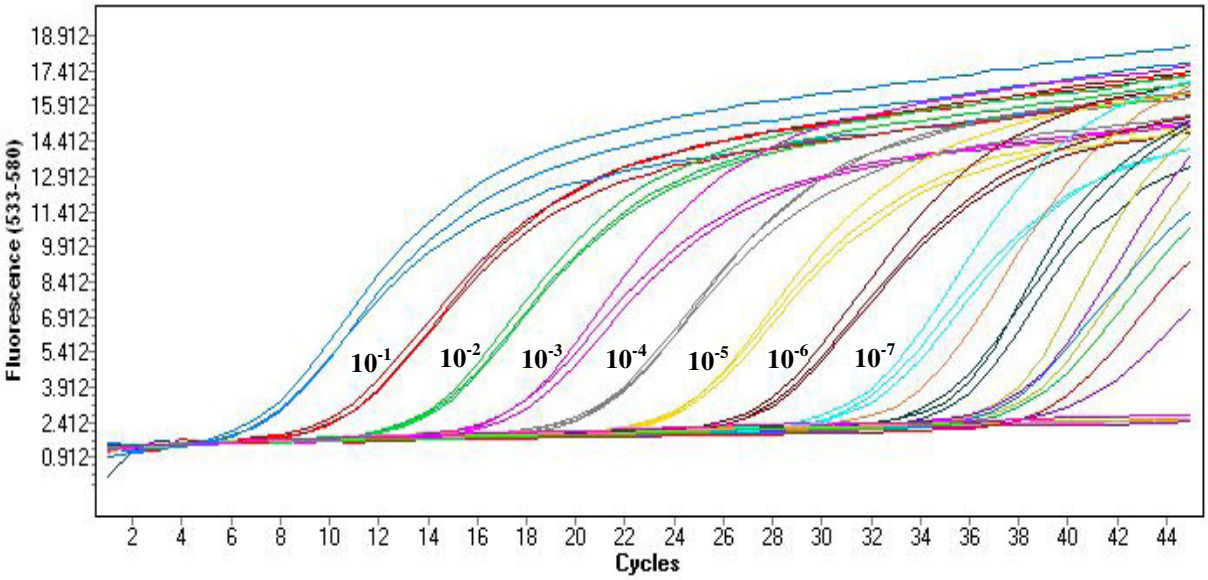

Figure 6.1: **The amplification curves of *A. vaginae* standards, including serial dilutions 10^{-1} to 10^{-7}**

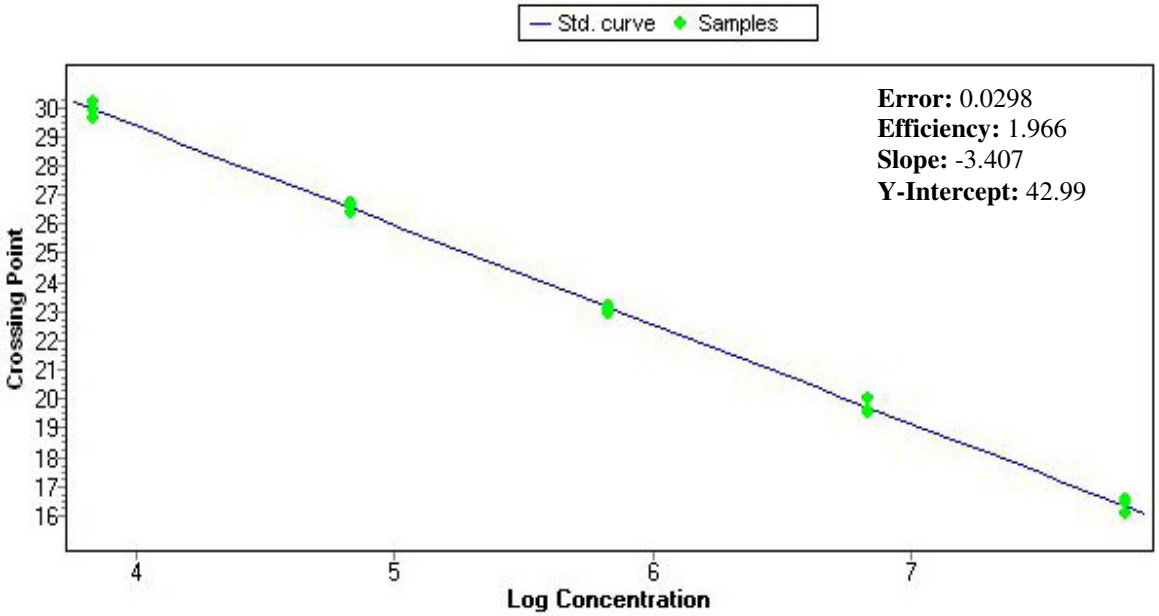

Figure 6.2: **The standard curve for *A. vaginae* generated from dilutions 10^{-3} to 10^{-7}. Quantification of clinical specimens was done based on an initial *A. vaginae* concentration of 6.77E+09 copies/reaction**

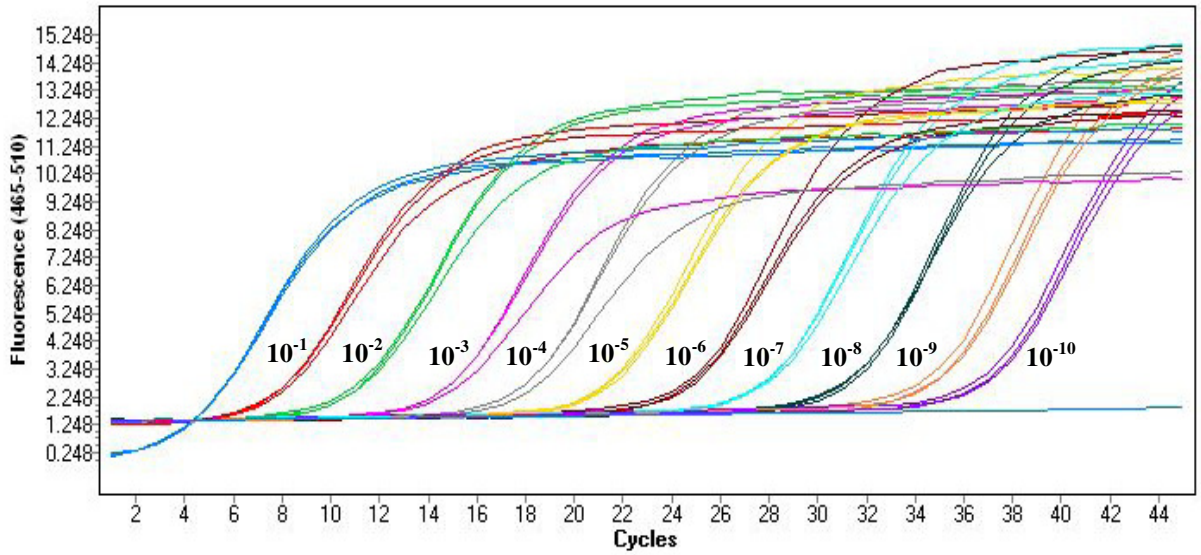

Figure 6.3: **The amplification curves of *G. vaginalis* standards, including serial dilutions 10^{-1} to 10^{-10}**

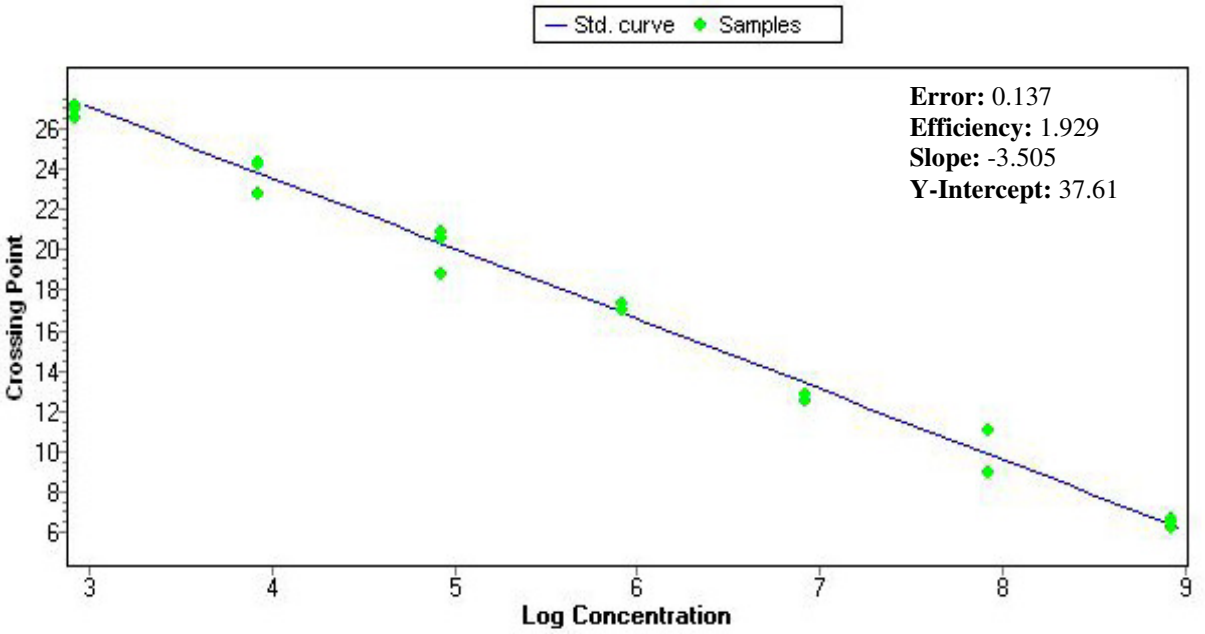

Figure 6.4: **The standard curve for *G. vaginalis* generated from dilutions 10^{-2} to 10^{-8}. Quantification of clinical specimens was done based on an initial *G. vaginalis* concentration of 8.35E+10 copies/reaction**

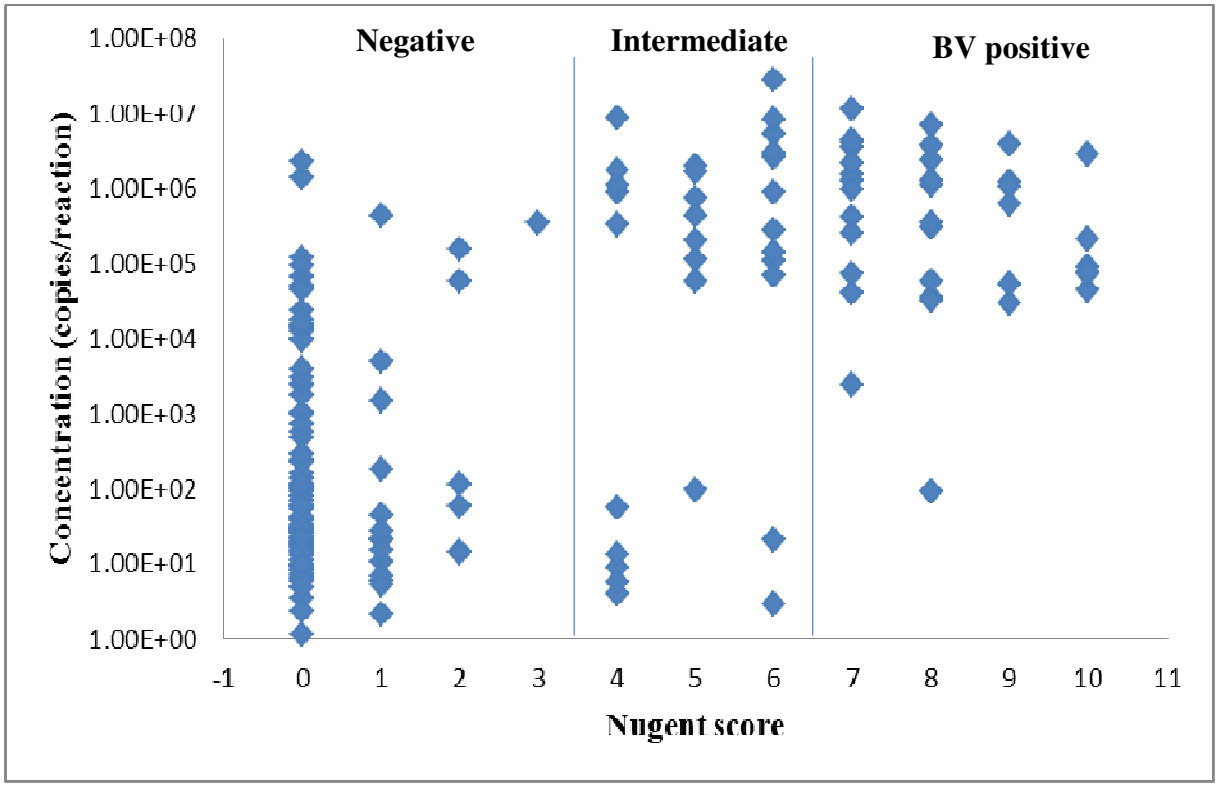

Figure 6.5: **Scatter plot showing the concentrations of *A. vaginae* in vaginal specimens, as determined by a qPCR assay, at different Nugent scores**

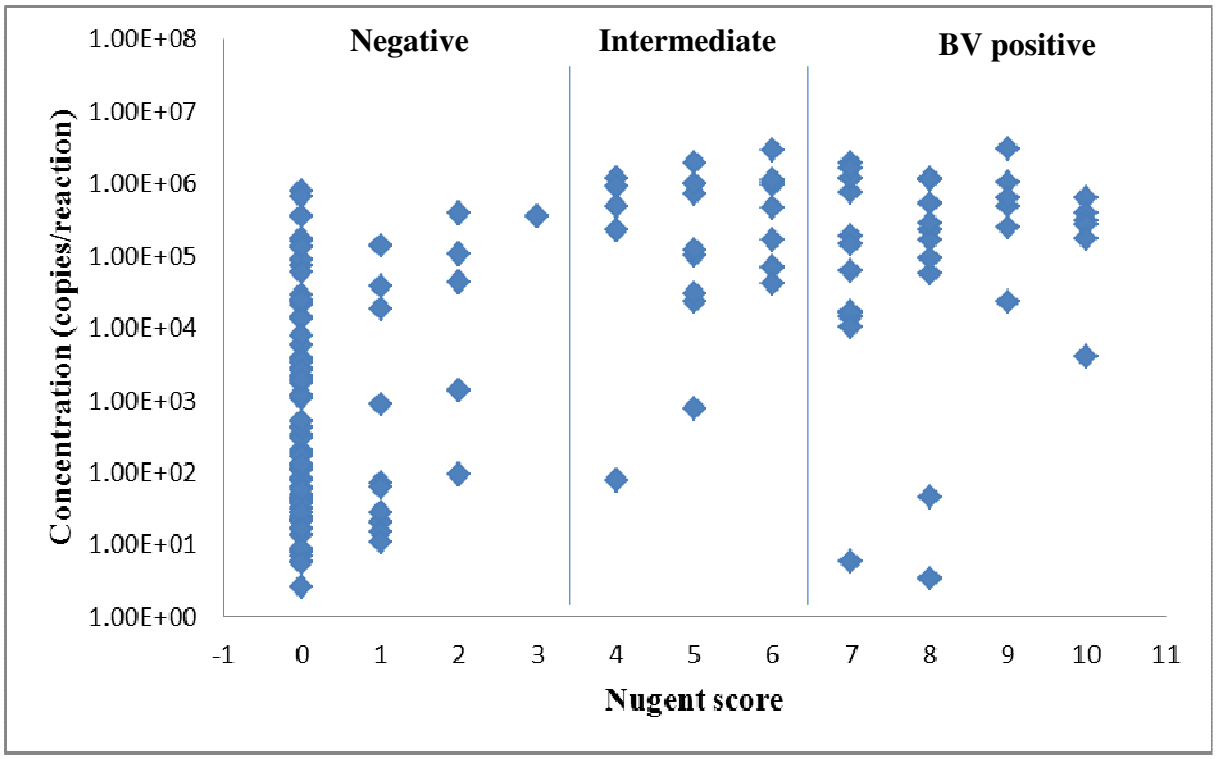

Figure 6.6: **Scatter plot showing the concentrations of *G. vaginalis* in vaginal specimens, as determined by a qPCR assay, at different Nugent scores**

CHAPTER 7

Concluding remarks

7.1 Conclusions

Bacterial vaginosis (BV) and genital mycoplasmas are among the most common reproductive tract infections (RTIs) in women worldwide (Myer *et al*., 2005; Patel and Nyirjesy, 2010). Numerous elements contribute and/or lead to the health issues that are often associated with these bacteria. These include the lack of accurate diagnostic methods, inadequate treatment and infection control strategies and increased antimicrobial resistance (Löfmark *et al*., 2010; Waites *et al*., 2012; Zankari *et al*., 2012).

The prevalence of BV and genital mycoplasmas in this study was high. With higher colonisation/infection rates, the risk of ascending infections and eventually poor pregnancy outcomes are inevitably higher. High prevalence rates may partly be due to shortcomings of syndromic treatment, an approach followed in the South African health sector (Johnson *et al*., 2011). These include (i) non-compliance of taking drugs by patients, (ii) non-adherence to or incorrect application of RTI treatment guidelines by health-care workers, (iii) the misinterpretation of specific syndromes by health-care workers and (iv) RTI drug shortages (Romoren *et al*., 2007; Johnson *et al*., 2011). This may lead to a rise in the number of insufficient prescriptions and untreated infections. Another contributing factor to the high prevalence of especially BV may be the high number of ethnic black patients included in this study (approximately 80% to 90%). It is reported that black ethnicity is one of the strongest correlates of BV (Cherpes *et al*., 2008; Allsworth and Peipert, 2011). Even though most of these patients were mostly South African nationals, a large proportion of patients were foreign nationals (mainly Nigerian, Zimbabwean and Congolese).

The shortcomings of syndromic management necessitate the evaluation of other treatment or management strategies. Romoren and colleagues (2007) studied antenatal care attendees in Botswana and found that the clinical screening for signs of vaginal discharge at the initial antenatal care visit were more effective than the mere use of symptoms as an entry point for metronidazole treatment. Romoren *et al*. (2007) confirmed that symptoms are poor predictors of the aetiological agents responsible for infection and emphasised the benefit of screening for

RTIs. The use of syndromic approaches may have superseded the investigation of the use of point-of-care (POC) tests for the screening of common RTIs, such as BV and genital mycoplasmas (Romoren *et al*., 2007). In South Africa there is a need for simple, fast and reliable diagnostic tests.

The 54.9% contamination rate on A2 agar media found in this study highlights the low sensitivity of culture methods that are routinely used for the detection of genital mycoplasmas. Nonetheless, this method of detection is still considered the gold standard method of diagnosing mycoplasma infections. A possibility for genital mycoplasmas is to either improve or modify conventional culture methods, such as A2 agar medium to make it more selective for genital mycoplasmas and enhance growth. However, this would still require skilled laboratory personnel due to the fastidious nature of genital mycoplasmas. Alternatively, a more expensive but newer agar medium, such as A7 agar medium can be used. Many genital mycoplasma 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 mPCR assay detected more genital mycoplasmas than the A2 agar medium, which reiterates the higher sensitivity of a molecular technique. The comparison between the mPCR assay and the Mycofast Revolution assay indicated that the Mycofast Revolution assay may 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.

The Nugent score results correlated with the qPCR assay used for the detection of *A. vaginae* and *G. vaginalis* as specimens with high Nugent scores were found to contain high concentrations of *G. vaginalis* by the qPCR assay. The threshold values of *A. vaginae* and *G. vaginalis* established by the qPCR assay can be used to predict BV when only a molecular assay is being used. Nonetheless, the qPCR assay and the Nugent scoring system can rarely be used in a clinical setup to diagnose a patient with BV. Amsel's criteria, although subjective, is a more practical test that can be used by clinicians to diagnose BV (Romoren *et al*., 2007). This test is cheap and can be done at the bedside of the patient, with or without the aid of a microscope (Schoeman, 2002). However, to exclude the ambiguity of results obtained with this method and increase the specificity of diagnosis, a more definitive

approach is needed (Hay, 2010). The Ison and Hay criteria, a simplified scoring system, is a more advantageous method and has also been recommended for use in genitourinary medicine clinics in the United Kingdom for BV diagnosis (Hay, 2010).

Diagnostic tests are essential for the effective management of RTIs. Following the findings of the present study, it is recommended that patients be screened at the first antenatal clinic visit for BV. The screening of women for BV and genital mycoplasmas at the first antenatal visit may identify asymptomatic cases and correctly identify symptomatic cases. This may be followed by the use of antimicrobial agents as prescribed by the syndromic management guidelines, given the correct syndrome is identified. Moreover, screening and subsequent treatment of BV may reduce women's susceptibility to genital mycoplasma and HIV infections as these infections are often associated with BV (Myer *et al*., 2005). The findings of the present study confirmed the association of *M. hominis* and *U. parvum* with BV. Although there is no clear evidence of the advantage for the screening or treatment of all women who have asymptomatic BV, evidence suggests that screening and treatment in pregnant women may reduce preterm birth (PTB) and low birth weight (LBW) infants (Farquharson *et al*., 2005; Swadpanich *et al*., 2008). It is suggested that a cost-effective approach is followed by using pH-sensitive paper to measure vaginal pH. A pH value of less than 4.5 almost excludes BV infection (Hay, 2010). If the pH is high, a vaginal swab can be sent to the laboratory for examination by Gram-staining and subsequent Ison and Hay grading. If further analyses are necessary, a qPCR assay can be used. It is also recommended that if the clinician suspects a genital mycoplasma infection, the specimens should be screened with the Mycofast Revolution assay.

7.2 Future research

The findings of this study lends to the continued investigation of affordable, rapid and accurate diagnostic tests for the detection of BV and genital mycoplasmas. Sensitive molecular assays, such as mPCR and qPCR assays are good assays for research purposes and can be used to evaluate the performance of other tests.

The confirmation that high concentrations of *A. vaginae* and *G. vaginalis* are significantly present in specimens with high Nugent scores places the focus on these two bacterial species for BV research. The discoveries made by Swidsinski *et al*. (2010) that some *G. vaginalis*

produce biofilms and that these biofilms are established on the endometrium of the female reproductive tract, a site previously thought to be sterile, paved the way for organism-specific research for the pathogenesis of BV. To identify the genes responsible for biofilm formation would be obvious strategies to create models for treatment and intervention. *In vitro* data showed that particular probiotic lactobacilli have the potential to reduce biofilm formation (Verstraelen and Swidsinski, 2013). Nevertheless, an *in vitro* model of the BV biofilm is needed for research into this subject.

Further investigation on the genes and mechanisms of antimicrobial resistance of genital mycoplasmas is needed. Genetic analyses of the genes conferring antimicrobial resistance may give a better indication 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). The *Mycoplasma* genus is known for phase switching of genes that express surface membrane proteins (Yogev *et al*., 2002; Citti *et al*., 2010). Phase switching allows host adaptation and plays an important role in evading the immune responses to sustain diversity in the proliferating population (Zimmerman *et al*., 2011). The genes that are prone to point mutations and phase switching may be used as models and serve as targets to study the mechanisms of conferring resistance.

The epidemiology of BV and genital mycoplasmas is still poorly understood. The discovery of gene mutations, biofilm formation and the classification of new vaginal bacteria, such as BV-associated bacteria (BVAB) 1, 2 and 3 prove that some progress has been made. At the same time, these findings pose more questions and opens up a whole new era for BV and genital mycoplasma research.

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APPENDIX A

Reagents, buffers and gels used in experimental procedures

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

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

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

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

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

Dissolve 54 g of Tris-base and 27.5 g of boric acid in 800 ml of distilled water and add 20 ml of 0.5 M EDTA buffer. Adjust the pH to 8.2 and bring the volume to 1 L. Autoclave at 121ºC for 15 min

3. Brain Heart Infusion (BHI) broth (500 ml)

BHI powder (Merck, Germany)	18.5	g
Distilled water	400	ml

Dissolve 18.5 g of BHI broth in 400 ml of distilled water and bring the volume to 500 ml. Autoclave at 121ºC for 15 min

4. Agarose gels

4.1 Low Electroendosmosis (LE) agarose gel

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

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

4.2 MetaPhor agarose gel

MetaPhor agarose powder (Lonza, USA)	2	g
TBE buffer	100	ml
Ethidium bromide (10 mg/ml) (Promega, USA)	5	µl

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

APPENDIX B

Experimental procedures

1. Deoxyribonucleic acid (DNA) extraction

1. Bacterial DNA was isolated with the ZR Fungal/Bacterial DNA kit (Zymo Research, USA) from modified Amies transport medium of obtained vaginal swabs.
2. One hundred and fifty microliters of the Amies transport medium was added to a ZR BashingBead Lysis Tube (Zymo Research, USA) together with 750 µl Lysis solution.
3. The tube was vortexed (VELP Scientifica, Italy) for ± 5 min at maximum speed.
4. The ZR BashingBead Lysis Tube (Zymo Research, USA) was centrifuged in a Spectrafuge centrifuge (Labnet International, USA) at ≥10 000 *x g* for 1 min (± 25ºC).
5. Up to 400 µl of the supernatant was transferred to a Zymo-spin IV Spin Filter in a collection tube and centrifuged (Spectrafuge centrifuge; Labnet International, USA) at 4 500 *x g* for 1 min (± 25ºC).
6. To the filtrate in the collection tube, 1 200 µl of bacterial DNA binding buffer was added.
7. A volume of 800 µl was transferred to a Zymo-Spin IIC column (Zymo Research, USA) in a collection tube and centrifuged (Spectrafuge centrifuge; Labnet International, USA) at 10 000 *x g* for 1 min (± 25ºC).
8. The flow-through from the collection tube was discarded and the latter step repeated.
9. Two hundred microlitres of DNA Pre-Wash Buffer (Zymo Research, USA) was added to the Zymo-spin IIC column (Zymo Research, USA) in a new collection tube and centrifuged (Spectrafuge centrifuge; Labnet International, USA) at 10 000 *x g* for 1 min.
10. A volume of 500 µl of bacterial DNA wash buffer was added to the Zymo-spin IIC column (Zymo Research, USA) and centrifuged (Spectrafuge centrifuge; Labnet International, USA) at 10 000 *x g* for 1 min (± 25ºC).
11. The Zymo-spin IIC column (Zymo Research, USA) was transferred to a clean 1.5 ml micro centrifuge tube and 100 µl DNA elution buffer (Zymo Research, USA) was added to the column matrix and centrifuged (Spectrafuge centrifuge; Labnet International, USA) at 10 000 *x g* for 30 s to elute the DNA (± 25ºC).
12. The eluted ultra-pure DNA was stored at -20ºC until further analysis.

2. Gram-staining and the grading of Gram-stained vaginal smears according to the Nugent scoring system (Nugent *et al*., 1991; Madigan and Martinko, 2006)

1. Vaginal smears were fixed to slides by heating for 5 s using a Bunsen burner.
2. Gram staining was performed by flooding the heat-fixed smear with crystal violet (Diagnostic Media Products, NHLS, South Africa) for 1 min.
3. Excess crystal violet was rinsed off with water for 10 s.
4. Iodine solution (Diagnostic Media Products, NHLS, South Africa) was added for 1 min.
5. Excess iodine was rinsed off with water for 10 s.
6. Decolourisation with ethanol (Merck, Germany) was done for ± 30 s.
7. Excess ethanol was rinsed off with water for 10 s.
8. Bacterial cells were counterstained with safranin (Diagnostic Media Products, NHLS, South Africa) for 1 min.
9. Excess safranin was rinsed off with water for 10 s.
10. Gram-stained slides were dried with blotting paper.
11. Gram-stained slides were examined with a microscope (Zeiss, Germany) for three different morphotypes:
    a) *Lactobacillus* species – large, sometimes long Gram-positive rods
    b) *Gardnerella vaginalis/Bacteroides* – small Gram-variable/Gram-negative rods (these two morphotypes are combined due to their similar appearance on Gram stain)
    c) *Mobiluncus* – curved Gram-negative/Gram-variable rods
12. Five oil immersion fields per slide were examined for the presence of these three different morphotypes.
13. Each slide 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 1).
14. A Gram-stain score (a number between 0 and 10) was calculated by adding the assigned score according to Table 1.
15. 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.

Table 1: Nugent scoring system (0 to 10) for Gram-stained vaginal smears[1] (Nugent *et al*., 1991)

Score	*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.

REFERENCES

Madigan MT, Martinko JM (2006) Brock: Biology of Microorganisms. 11th ed. Upper Saddle River: Prentice Hall

Nugent RP, Krohn MA, Hillier SL (1991) Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. *Journal of Clinical Microbiology* **29**:297-301

APPENDIX C: Clinical data of patients and results for diagnostic tests performed

Table 2: Clinical information of low-risk patients and combined results including Nugent score results, mPCR assay results and qPCR assay concentrations of *G. vaginalis* and *A. vaginae*

Sample number	Age	Gestational age of current pregnancy	Possible risk factor	HIV status	BV status	BV-associated bacteria		Genital mycoplasmas			
						Concentration of AV	Concentration of GV	MH	MG	UU	UP
A1	20	25	Asthma	-	+	2.98E+04	2.42E+05	-	-	-	+
A2	38	32	AMA, Diabetes, Epilepsy	-	I	9.12E+00	-	-	-	-	-
A3	32	29	Fibroids	NA	-	3.06E+01	4.26E+02	-	-	-	+
A6	37	21	AMA, C/S	-	-	2.15E+01	7.09E+01	-	-	-	-
A7	30	30	None identified	+	-	-	-	+	-	-	+
A8	31	31	Bicornuate uterus, HEELP syndrome	-	I	-	2.24E+04	-	-	-	-
A9	23	20	Epilepsy	-	+	2.46E+03	1.65E+04	-	-	-	+
A12	40	35	AMA, Asthma, Diabetes	-	+	2.81E+06	6.14E+05	+	-	-	+
A13	22	38	None identified	-	-	2.10E+01	-	+	-	-	+
A15	22	26	*Candida* Infection, Cardiac	-	-	8.84E+00	8.01E+01	-	-	-	+
A16	22	39	UTI	-	-	3.56E+05	3.44E+05	-	-	-	+
A17	39	11	AMA, Fibroids	-	+	4.39E+04	3.94E+03	+	-	-	+

AMA : Advanced maternal age
AV : *Atopobium vaginae*
C/S : Previous caesarean section
GV : *Gardnerella vaginalis*
I : Intermediate
I/C : Inconclusive
IUD : Intrauterine device
MG : *Mycoplasma genitalium*
MH : *Mycoplasma hominis*
NA : Not available/unknown
NVD : Normal vaginal delivery
PET : Pre-eclampsia toxin
UP : *Ureaplasma parvum*
UTI : Urinary tract infection
UU : *Ureaplasma urealyticum*
- : Negative
+ : Positive

Table 2: Clinical information of low-risk patients and combined results including Nugent score results, mPCR assay results and qPCR assay concentrations of *G. vaginalis* and *A. vaginae* (continued)

Sample number	Age	Gestational age of current pregnancy	Possible risk factor	HIV status	BV status	BV-associated bacteria		Genital mycoplasmas			
						Concentration of AV	Concentration of GV	MH	MG	UU	UP
A18	20	39	Breech (Archondo plasia)	-	-	1.39E+02	3.58E+03	+	-	-	+
A19	25	35	Fibroids, Right inguinal hernia	-	I	2.90E+00	-	-	-	-	-
A20	22	39	Cardiac, Hypertension	NA	+	7.46E+04	1.38E+04	+	-	-	+
A21	20	38	Kidney related diseases	-	-	-	4.45E+01	-	-	-	-
A23	30	29	Fibroids	-	-	2.19E+00	-	-	-	-	+
A24	32	9	Hypertension	NA	-	1.82E+01	7.21E+04	-	-	-	-
A25	23	38	C/S, PET	-	-	1.01E+01	-	-	-	-	+
A27	25	37	Cardiac	-	-	3.56E+00	-	-	-	-	+
A28	35	14	AMA, Rheumatoid Arthritis	+	-	1.55E+05	3.77E+05	+	-	-	+
A29	35	31	AMA, Asthma, Fibroids	-	-	4.29E+05	1.37E+05	+	-	-	+

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- : Negative
+ : Positive

Table 2: Clinical information of low-risk patients and combined results including Nugent score results, mPCR assay results and qPCR assay concentrations of *G. vaginalis* and *A. vaginae* (continued)

Sample number	Age	Gestational age of current pregnancy	Possible risk factor	HIV status	BV status	BV-associated bacteria		Genital mycoplasmas			
						Concentration of AV	Concentration of GV	MH	MG	UU	UP
A30	34	32	Hypertension, Thyroid related problems	-	-	-	3.93E+01	+	-	-	+
A31	33	17	None identified	-	-	4.23E+01	3.20E+02	+	-	-	+
A32	21	24	Anti-Rho antibodies	-	-	4.95E+03	3.73E+04	+	-	-	-
A33	33	33	Epilepsy	-	-	1.18E+02	1.33E+03	-	+	-	-
A34	23	37	Kidney related diseases	+	-	6.57E+04	2.27E+04	+	-	-	+
A35	29	30	C/S, Mood disorder	-	-	1.18E+05	7.79E+05	-	+	-	+
A36	34	36	Diabetes	-	-	6.96E+01	-	+	-	-	+
A37	37	40	Diabetes, AMA	-	-	3.73E+01	-	-	-	-	-
A38	33	33	Fibroids, DVT	-	-	2.39E+02	3.88E+01	-	-	-	+
A39	30	5	C/S	NA	+	1.14E+07	-	-	-	-	+
A40	28	13	DVT	+	+	7.33E+04	3.87E+05	+	-	-	+

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NVD : Normal vaginal delivery
PET : Pre-eclampsia toxin
UP : *Ureaplasma parvum*
UTI : Urinary tract infection
UU : *Ureaplasma urealyticum*
- : Negative
+ : Positive

Table 2: Clinical information of low-risk patients and combined results including Nugent score results, mPCR assay results and qPCR assay concentrations of *G. vaginalis* and *A. vaginae* (continued)

Sample number	Age	Gestational age of current pregnancy	Possible risk factor	HIV status	BV status	BV-associated bacteria		Genital mycoplasmas			
						Concentration of AV	Concentration of GV	MH	MG	UU	UP
A41	39	17	Hypertension, AMA, Thyroid related problems	-	-	3.80E+03	1.98E+03	-	-	-	+
A43	24	30	Kidney related diseases	-	I	4.08E+00	-	-	+	-	+
A44	23	33	Epilepsy	-	-	1.41E+04	1.21E+03	+	-	-	-
A45	24	25	Thyroid related problems	-	-	2.12E+01	-	-	-	-	+
A46	26	17	C/S, Hypertension, Twin pregnancy, Inadequate space for twin babies	-	-	2.29E+03	1.71E+05	-	-	-	-
A47	29	32	Twins Pregnancy, Thyroid related problems	-	-	3.05E+01	-	-	-	-	+
A48	28	32	Diabetes	-	+	2.57E+05	1.61E+04	+	-	-	+
A49	29	32	Diabetes, C/S	-	-	1.06E+03	2.84E+03	-	-	-	+
A50	33	25	Diabetes, C/S	-	I	2.85E+07	9.47E+05	-	-	-	+

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Table 2: Clinical information of low-risk patients and combined results including Nugent score results, mPCR assay results and qPCR assay concentrations of *G. vaginalis* and *A. vaginae* (continued)

Sample number	Age	Gestational age of current pregnancy	Possible risk factor	HIV status	BV status	BV-associated bacteria		Genital mycoplasmas			
						Concentration of AV	Concentration of GV	MH	MG	UU	UP
A51	31	22	DVT	+	I	8.96E+05	2.25E+05	+	-	-	-
A52	24	37	CMV infection	-	-	1.12E+02	-	+	-	-	-
A53	38	22	Twin pregnancy, AMA	-	-	3.97E+01	1.25E+02	+	-	-	+
A54	29	33	Kidney related diseases, hydronephrosis	-	I	-	-	+	-	-	+
A55	22	37	Cardiac	+	-	1.47E+01	1.03E+05	+	-	-	+
A56	33	20	Cardiac	-	+	3.82E+06	1.13E+06	+	-	-	+
A57	22	37	Cardiac	-	I	5.45E+06	2.82E+06	+	-	-	+
A58	42	30	C/S, AMA	-	+	1.50E+06	7.56E+05	+	-	-	+
A59	40	25	AMA, Endometriosis	NA	-	9.69E+00	-	-	-	-	-
A60	22	24	Diabetes	-	-	-	-	-	-	-	+
A61	30	35	Cardiac, Hypertension	-	-	5.88E+01	-	+	-	-	+
A62	27	22	Rhesus negative	+	I	8.87E+06	1.15E+06	+	-	-	-
A63	25	23	Hypertension	-	+	3.66E+06	1.95E+06	+	-	-	-
A64	31	35	Cardiac, Hypertension	+	I	2.82E+05	1.01E+06	+	-	-	+

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Table 2: Clinical information of low-risk patients and combined results including Nugent score results, mPCR assay results and qPCR assay concentrations of *G. vaginalis* and *A. vaginae* (continued)

Sample number	Age	Gestational age of current pregnancy	Possible risk factor	HIV status	BV status	BV-associated bacteria		Genital mycoplasmas			
						Concentration of AV	Concentration of GV	MH	MG	UU	UP
A65	21	34	Twin Pregnancy	-	-	-	7.78E+01	+	-	-	+
A66	29	24	Diabetes	-	-	2.18E+01	-	+	-	-	+
A67	31	29	Diabetes	-	-	-	-	-	-	-	+
A68	27	35	Cardiac	-	-	-	-	+	-	-	-
A69	34	35	Cardiac	-	-	-	8.07E+01	+	-	-	-
A70	25	29	Cardiac	-	-	4.77E+02	1.96E+02	+	-	-	+
A71	28	11	Diabetes, Twin pregnancy, Thalassemia	NA	-	-	8.02E+00	-	-	-	+
A72	38	35	Hypertension, Twin pregnancy, AMA	-	-	3.94E+01	-	-	-	-	-
A73	37	30	AMA, C/S, Diabetes, Hypertension, PET	-	-	-	-	-	-	-	-
A74	34	26	Diabetes, C/S, Cardiac, Hypertension	+	+	2.16E+06	9.97E+03	+	+	-	-

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Table 2: Clinical information of low-risk patients and combined results including Nugent score results, mPCR assay results and qPCR assay concentrations of *G. vaginalis* and *A. vaginae* (continued)

Sample number	Age	Gestational age of current pregnancy	Possible risk factor	HIV status	BV status	BV-associated bacteria		Genital mycoplasmas			
						Concentration of AV	Concentration of GV	MH	MG	UU	UP
A75	23	29	*Candida* infection, Cardiac	-	-	3.00E+02	1.67E+03	+	-	-	+
A76	37	18	AMA, C/S, Hypertension	NA	-	9.79E+02	-	-	-	-	+
A77	25	27	Epilepsy	-	-	1.44E+04	2.08E+04	-	-	-	+
A78	32	28	Hypertension	-	-	1.28E+04	-	-	-	-	-
A79	34	23	Thyroid problems	-	-	-	8.00E+00	+	-	-	+
A80	22	19	Diabetes, Hypertension, Kidney related diseases	-	I	2.07E+05	3.01E+04	-	-	-	-
A81	33	17	Diabetes	+	-	-	-	+	-	-	+
A82	38	19	AMA	NA	I	2.61E+06	1.60E+05	+	-	-	+
A83	25	23	Autoimmune disease, Hypertension, PET	-	+	3.61E+04	5.49E+04	-	-	-	+
A84	22	14	Endocrine	-	-	1.78E+01	1.11E+02	+	-	-	-

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Table 2: Clinical information of low-risk patients and combined results including Nugent score results, mPCR assay results and qPCR assay concentrations of *G. vaginalis* and *A. vaginae* (continued)

Sample number	Age	Gestational age of current pregnancy	Possible risk factor	HIV status	BV status	BV-associated bacteria		Genital mycoplasmas			
						Concentration of AV	Concentration of GV	MH	MG	UU	UP
A85	23	10	Hypertension, Kidney related disease, APLS, CREST syndrome, Previous HGSIL	-	+	5.31E+04	2.24E+04	+	-	-	+
A86	21	18	Thalassemia	NA	-	9.80E+03	1.40E+04	-	-	+	+
A87	22	24	Oligohydramnios, Distended bladder on Foetus	-	-	9.40E+00	-	-	+	-	+
A88	37	24	Diabetes, Hypertension, AMA	NA	+	2.10E+05	3.03E+05	+	-	-	+
A89	39	18	AMA	+	-	8.00E+01	5.04E+01	-	+	-	+
A90	42	14	AMA	-	-	1.07E+01	1.99E+01	+	+	-	+
A91	27	28	None identified	-	-	-	1.83E+04	-	-	-	+
A92	36	27	AMA	-	-	6.00E+01	1.82E+02	+	-	-	+
A93	26	26	Twin pregnancy	-	I	1.08E+05	6.96E+04	+	-	-	+
A94	24	23	Epilepsy, Rhesus negative	-	-	-	1.27E+02	+	-	-	+

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Table 2: Clinical information of low-risk patients and combined results including Nugent score results, mPCR assay results and qPCR assay concentrations of *G. vaginalis* and *A. vaginae* (continued)

Sample number	Age	Gestational age of current pregnancy	Possible risk factor	HIV status	BV status	BV-associated bacteria		Genital mycoplasmas			
						Concentration of AV	Concentration of GV	MH	MG	UU	UP
A95	33	23	None identified	NA	I	1.99E+06	7.06E+05	+	-	-	+
A96	22	19	Myasthenia gravis	-	+	4.42E+06	1.19E+06	+	+	+	+
A97	32	17	Thyroid related problems	-	-	-	2.77E+01	+	-	-	+
A98	23	31	Twin pregnancy	-	-	5.71E+02	1.02E+03	+	-	-	+
A99	36	20	C/S, AMA	+	I	1.64E+06	1.90E+06	+	+	-	+
A100	22	29	Twin pregnancy	+	I	2.99E+06	4.60E+05	+	+	-	+
A101	42	40	C/S, AMA	NA	-	2.92E+01	2.07E+01	+	+	-	+
A102	19	31	Twin pregnancy	-	-	1.01E+01	2.81E+01	+	+	-	+
A103	22	31	Diabetes	-	-	-	3.14E+01	+	+	-	+
A104	31	21	None identified	-	-	8.23E+00	1.37E+01	+	+	-	+
A105	41	30	Diabetes, C/S, Hypertension, PET, AMA,	-	-	-	-	+	-	-	+
A106	26	24	Cardiac	-	+	1.23E+06	3.01E+06	+	-	-	+
A107	22	20	Cardiac	-	+	6.22E+05	1.03E+06	+	-	-	-
A108	25	35	Cardiac	+	+	2.35E+06	1.15E+06	+	+	-	+
A109	22	34	PV Bleeding	-	-	5.05E+00	2.64E+00	-	-	-	+

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Table 2: Clinical information of low-risk patients and combined results including Nugent score results, mPCR assay results and qPCR assay concentrations of *G. vaginalis* and *A. vaginae* (continued)

Sample number	Age	Gestational age of current pregnancy	Possible risk factor	HIV status	BV status	BV-associated bacteria		Genital mycoplasmas			
						Concentration of AV	Concentration of GV	MH	MG	UU	UP
A110	28	17	Rheumatoid arthritis	-	-	9.68E+03	5.64E+03	-	+	-	+
A111	30	27	Hepatomegaly	-	-	2.26E+02	4.30E+01	+	-	-	+
A112	29	16	Hypertension, Thyroid related diseases	-	+	3.88E+06	4.75E+05	-	+	-	+
A113	43	13	AMA, Pulmonary embolism	-	+	3.02E+05	-	+	-	-	+
A114	32	23	Twin pregnancy	-	-	-	1.51E+01	+	-	-	-
A115	27	23	Twin pregnancy	-	-	8.17E+00	8.54E+04	+	-	-	+
A116	22	32	Diabetes	-	-	0	-	-	-	-	+
A117	40	23	AMA, C/S	+	I	5.76E+04	1.00E+05	+	-	-	+
A119	43	33	AMA	+	-	9.98E+01	-	-	-	-	-
A121	22	12	Epilepsy	-	+	1.27E+06	1.17E+06	-	-	-	+
A122	31	33	Cardiac, Tuberculosis	+	-	2.89E+02	3.80E+03	+	-	-	+
A123	24	31	C/S, Cardiac, Asthma	-	-	9.18E+01	6.26E+01	+	-	-	+
B69	33	21	Diabetes	-	-	2.94E+01	2.38E+01	+	-	-	+

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UTI : Urinary tract infection
UU : *Ureaplasma urealyticum*
- : Negative
+ : Positive

Table 3: **Clinical information of high-risk patients and combined results including Nugent score results, mPCR assay results and qPCR assay concentrations of *G. vaginalis* and *A. vaginae***

Sample number	Age	Gestational age of current pregnancy	No. of pregnancy losses	Gestational age of pregnancy loss(es) (weeks)	High-risk factor identified	HIV status	BV status	BV-associated bacteria		Genital mycoplasmas			
								Concentration of AV	Concentration of GV	MH	MG	UU	UP
B1	28	18	3	8 w; 26 w; 26 w	POH	-	+	3.31E+04	2.22E+05	+	-	-	+
B2	26	38	3	8 w; 8 w; 16 w	POH	+	-	1.78E+03	5.79E+04	+	+	-	+
B3	33	29	2	2 x Between 16w and 27w	POH, Incompetent Cervix	-	-	-	-	-	-	-	+
B4	37	31	3	Ectopic (NA); 16 w; 24 w	POH, Hypertension, PET	-	-	2.39E+00	-	-	-	-	-
B5	27	19	5	1 x <15 weeks; 40 w	POH	+	+	4.01E+04	1.86E+05	+	-	-	-
B6	33	29	3	1x Between 16-27w; 2x Between 28-36w	POH, Hypertension	+	-	1.44E+01	9.28E+01	-	-	-	-
B7	27	38	1	1 x Between 16-27	POH, Alcohol usage	-	-	-	-	-	-	-	-
B8	29	38	1	12 w	POH, C/S	-	-	-	-	-	-	-	+
B9	30	9	2	12 w; 12 w	POH, Hypertension	+	+	9.36E+01	4.60E+01	+	-	-	+

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Table 3: Clinical information of high-risk patients and combined results including Nugent score results, mPCR assay results and qPCR assay concentrations of *G. vaginalis* and *A. vaginae* (continued)

Sample number	Age	Gestational age of current pregnancy	No. of pregnancy losses	Gestational age of pregnancy loss(es) (weeks)	High-risk factor identified	HIV status	BV status	BV-associated bacteria		Genital mycoplasmas			
								Concentration of AV	Concentration of GV	MH	MG	UU	UP
B10	29	26	2	20 w; 20 w	POH	-	+	3.42E+05	3.37E+00	-	-	-	+
B11	26	26	7	8 w; 8 w; 16 w; 20 w; 24 w; 24 w; 28 w	POH	+	-	4.45E+01	-	+	-	-	+
B12	39	16	4	16 w; 16 w; 20 w; 20 w	POH	-	+	6.91E+06	5.31E+05	-	-	-	+
B13	39	24	3	12 w; 12 w; 14 w	POH	NA	+	1.10E+06	1.67E+05	+	-	-	+
B14	23	8	3	1 x <15 w; 6 w; 8 w	POH	-	+	3.78E+06	2.82E+05	+	-	-	+
B15	35	26	3	40 w; 40 w; 40 w	POH, C/S, AMA	+	-	1.96E+01	-	-	-	-	+
B16	34	36	3	1 x <15 w; 20 w; 24 w	POH, Thyroid related problems, Rhesus negative	-	-	-	-	-	-	-	-
B17	30	23	3	12 w; 12 w; 40 w	POH	-	-	9.44E+00	-	+	-	-	+

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Table 3: Clinical information of high-risk patients and combined results including Nugent score results, mPCR assay results and qPCR assay concentrations of *G. vaginalis* and *A. vaginae* (continued)

Sample number	Age	Gestational age of current pregnancy	No. of pregnancy losses	Gestational age of pregnancy loss(es) (weeks)	High-risk factor identified	HIV status	BV status	BV-associated bacteria		Genital mycoplasmas			
								Concentration of AV	Concentration of GV	MH	MG	UU	UP
B18	32	35	2	8 w; 12 w	POH, Rhesus negative	-	-	5.07E+04	3.53E+05	+	-	-	+
B19	28	33	1	15 w	POH, Thyroid related problems	-	+	-	-	-	-	-	+
B20	22	30	1	12 w	POH, Placental growth	-	-	2.34E+04	2.88E+04	+	+	-	+
B21	37	18	3	8 w; 12 w; 12 w	POH, AMA	+	+	9.00E+04	2.65E+05	+	-	-	-
B22	38	34	4	3 x <15 w; 6 w	POH, C/S, AMA	-	-	1.29E+01	4.28E+01	-	-	-	+
B23	33	37	1	8 w	POH, Hypertension	-	-	1.62E+02	2.93E+02	+	-	-	+
B24	38	35	3	1 x <15 w; 8 w; 11 w	POH, C/S, AMA	-	-	6.70E+00	-	-	-	-	+
B25	36	20	2	20 w; 24 w	POH, AMA	-	-	-	2.14E+03	-	-	-	+
B26	25	20	2	14 w; 24 w	POH	-	+	4.13E+05	1.40E+05	+	-	-	+

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Table 3: Clinical information of high-risk patients and combined results including Nugent score results, mPCR assay results and qPCR assay concentrations of *G. vaginalis* and *A. vaginae* (continued)

Sample number	Age	Gestational age of current pregnancy	No. of pregnancy losses	Gestational age of pregnancy loss(es) (weeks)	High-risk factor identified	HIV status	BV status	BV-associated bacteria		Genital mycoplasmas			
								Concentration of AV	Concentration of GV	MH	MG	UU	UP
B27	27	12	3	7 w; 9 w; 16 w	POH, Incompetent cervix	-	-	-	-	-	-	-	-
B28	28	30	2	24 w; 24 w	POH, Incompetent cervix	-	I	3.27E+05	7.59E+01	-	-	-	+
B29	29	31	4	16 w; 16 w; 20 w; 40 w	POH	+	I	1.14E+05	1.19E+05	-	-	-	+
B30	25	26	3	12 w; 20 w; 24 w	POH	-	-	2.70E+01	-	-	+	-	+
B31	43	37	2	END: 1day; 40 w	POH, AMA	-	I	6.98E+04	6.76E+04	-	-	-	+
B32	39	11	4	14 w; 20 w; 22 w; 22 w	POH, Hypertension, PET, AMA	-	I	2.16E+01	-	-	-	-	-
B33	21	12	1	29 w	POH, Epilepsy, Diabetes, Hypertension	-	+	-	1.62E+06	+	+	-	+
B34	29	34	1	24 w	POH, Diabetes, C/S	-	-	1.50E+03	8.85E+02	+	+	-	+

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Table 3: Clinical information of high-risk patients and combined results including Nugent score results, mPCR assay results and qPCR assay concentrations of *G. vaginalis* and *A. vaginae* (continued)

Sample number	Age	Gestational age of current pregnancy	No. of pregnancy losses	Gestational age of pregnancy loss(es) (weeks)	High-risk factor identified	HIV status	BV status	BV-associated bacteria		Genital mycoplasmas			
								Concentration of AV	Concentration of GV	MH	MG	UU	UP
B35	35	33	1	40 w	POH, Diabetes, C/S, AMA	+	I	5.64E+01	-	-	-	-	+
B36	35	32	2	8 w; 8 w	POH, Diabetes, AMA	+	+	4.04E+06	5.74E+00	+	+	-	+
B37	35	14	2	6 w; 20 w	POH, AMA, Incompetent cervix	-	-	1.53E+01	-	-	-	-	-
B38	36	24	2	12 w; 28 w	POH, C/S, Asthma, AMA	+	-	6.12E+00	-	-	-	-	-
B39	33	14	1	6 w	POH	-	+	1.08E+06	6.14E+05	+	-	-	+
B40	38	35	1	24 w	POH, Cardiac, AMA	-	I	-	7.58E+02	-	-	-	+
B41	32	33	2	8 w; 40 w	POH, Epilepsy	+	I	7.36E+05	9.67E+05	+	-	+	-
B42	27	28	2	9 w; 10 w	POH, Diabetes, Hypertension	NA	-	1.42E+06	2.40E+04	+	-	-	+
B43	30	29	1	17 w	POH, Diabetes	-	-	2.05E+01	-	+	-	-	+
B44	23	23	2	14 w; 20 w	POH	NA	-	6.89E+00	-	-	-	-	+

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Table 3: Clinical information of high-risk patients and combined results including Nugent score results, mPCR assay results and qPCR assay concentrations of *G. vaginalis* and *A. vaginae* (continued)

Sample number	Age	Gestational age of current pregnancy	No. of pregnancy losses	Gestational age of pregnancy loss(es) (weeks)	High-risk factor identified	HIV status	BV status	BV-associated bacteria		Genital mycoplasmas			
								Concentration of AV	Concentration of GV	MH	MG	UU	UP
B45	22	28	4	8 w; 8 w; 16 w; 40 w	POH	-	I	1.10E+06	9.14E+05	-	-	-	+
B46	38	15	1	6 w	POH, Diabetes, C/S, AMA	-	-	-	6.87E+00	-	+	-	+
B47	32	19	7	8 w; 8 w; 8 w; 16 w; 16 w; 16 w; 24 w	POH	-	-	-	-	-	-	-	-
B48	30	13	2	24 w; 32 w	POH	-	I	8.86E+05	1.11E+06	+	+	-	+
B49	27	31	2	2 x <15 w	POH, Cardiac	-	-	4.74E+00	4.07E+02	-	-	-	-
B50	25	12	1	24 w	POH, Cardiac	-	+	9.74E+05	6.03E+04	-	-	-	-
B51	21	21	1	9 w	POH, Cardiac	-	-	7.93E+01	1.69E+02	-	-	-	+
B52	31	24	1	1 x <15 w	POH, C/S, Hypertension, Thyroid related problems	-	-	-	-	-	-	-	-

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Table 3: Clinical information of high-risk patients and combined results including Nugent score results, mPCR assay results and qPCR assay concentrations of *G. vaginalis* and *A. vaginae* (continued)

Sample number	Age	Gestational age of current pregnancy	No. of pregnancy losses	Gestational age of pregnancy loss(es) (weeks)	High-risk factor identified	HIV status	BV status	BV-associated bacteria		Genital mycoplasmas			
								Concentration of AV	Concentration of GV	MH	MG	UU	UP
B53	33	25	1	16 w	POH, Twin pregnancy	NA	-	2.49E+03	1.32E+04	-	-	-	-
B54	26	11	1	24 w	POH, Diabetes, Twin pregnancy	-	-	-	-	-	-	-	+
B55	32	28	2	30 w; 34 w	POH	+	-	9.75E+04	1.58E+05	+	-	-	+
B56	30	23	6	7 w; 8 w; 12 w; 16 w; 16 w; 24 w	POH, Twin pregnancy	-	-	1.18E+02	5.09E+02	+	-	-	-
B57	36	27	1	1 x <15 w	POH, AMA, Rhesus negative	-	-	-	-	-	+	-	-
B58	35	18	3	8 w; 12 w; 12 w	POH, Epilepsy, AMA	NA	-	3.02E+03	7.82E+03	+	-	-	+
B59	27	34	1	20 w	POH, Thyroid related problems	-	-	1.85E+04	1.16E+03	+	+	-	-
B60	28	21	2	25 w; 35 w	POH	-	-	2.76E+01	5.76E+00	+	-	-	-

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Sample number	Age	Gestational age of current pregnancy	No. of pregnancy losses	Gestational age of pregnancy loss(es) (weeks)	High-risk factor identified	HIV status	BV status	BV-associated bacteria		Genital mycoplasmas			
								Concentration of AV	Concentration of GV	MH	MG	UU	UP
B61	25	22	3	1 x NA; 1 x <15 w; 12 w	POH	-	I	1.37E+01	-	+	-	-	-
B62	37	21	1	6 w	POH, AMA, Breech	-	-	1.54E+01	-	+	-	-	-
B63	21	26	1	10 w	POH	-	-	2.99E+01	8.55E+00	+	-	-	+
B64	42	22	2	36 w; 36 w	POH, AMA	+	-	1.52E+04	3.36E+03	-	-	-	+
B65	34	27		14 w	POH, Rhesus negative	NA	-	1.72E+01	-	+	+	-	-
B66	39	22	3	6 w; 9 w; 12 w	POH, AMA	NA	-	1.13E+01	2.17E+01	+	+	-	-
B67	31	25	4	7 w; 7 w; 9 w; 11 w	POH	-	-	5.26E+00	1.09E+01	-	-	-	-
B68	32	20		6 w; 10 w; 10 w; 11 w	POH, C/S	-	-	2.70E+01	-	-	-	-	-
B70	32	39	1	11 w	POH, Rhesus negative	+	-	1.46E+01	-	+	-	-	-
B71	32	31	1	1 x <15 w	POH	-	+	5.78E+04	1.14E+06	+	+	-	+
B72	29	34	1	24 w	POH	-	-	-	1.04E+02	-	-	-	+

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								Concentration of AV	Concentration of GV	MH	MG	UU	UP
B73	34	33	1	11 w	POH, Other	-	-	3.29E+01	2.16E+01	-	-	-	-
B74	26	26	1	16 w	POH, Thyroid related problems	-	-	2.30E+06	6.49E+05	+	-	-	+
B75	26	37	1	6 w	POH	-	I	1.76E+06	4.76E+05	+	-	-	-
B76	26	37	1	20 w	POH, Asthma	-	-	1.01E+02	1.70E+01	-	-	-	+
B77	24	35	1	8 w	POH, Asthma	-	-	7.24E+00	3.68E+01	+	-	-	+
B78	34	36	2	12 w; 24 w	POH, Fibroids	-	I	8.46E+06	-	-	-	-	+
B79	33	31	1	20 w	POH, PET	+	-	1.14E+02	1.17E+02	+	+	-	+
B80	26	24	2	10 w; 10 w	POH	+	-	4.39E+04	1.31E+05	+	-	+	+
B81	30	23	1	16 w	POH	-	-	-	2.73E+01	-	-	-	+
B82	34	28	2	16 w; 20 w	POH	-	I	1.43E+05	4.02E+04	+	-	-	+
B83	39	21	1	16 w	POH, C/S, Hypertension, PET, AMA	-	-	1.15E+00	5.62E+01	-	-	-	-
B84	40	25	1	12 w	POH, C/S, Cardiac, AMA	-	-	5.73E+04	4.25E+04	+	-	-	+

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Sample number	Age	Gestational age of current pregnancy	No. of pregnancy losses	Gestational age of pregnancy loss(es) (weeks)	High-risk factor identified	HIV status	BV status	BV-associated bacteria		Genital mycoplasmas			
								Concentration of AV	Concentration of GV	MH	MG	UU	UP
B85	41	37	4	2 x <15 w; 6 w; 6 w	POH, C/S, AMA, Regular thrush, Breech	-	-	-	-	-	-	-	+
B86	30	38	1	12 w	POH, Twin pregnancy	-	-	3.92E+03	2.55E+03	-	+	-	+
B87	26	32	1	15 w	POH, C/S	-	-	-	-	+	-	-	+
B88	21	16	2	8 w; 9 w	POH, Rhesus negative, Cystic Ovaries	-	-	2.26E+01	-	-	-	-	+
B89	30	33	2	8 w; 40 w	POH, C/S, Hypertension, Cystic Ovaries	-	-	-	-	+	-	-	-
B90	29	14	4	1 x <15 w; 7 w; 9 w; 9 w	POH, PV bleeding	-	-	-	1.25E+02	-	-	-	+
B91	30	14	2	12 w; 12 w	POH, Epilepsy, Hypertension	-	-	5.44E+01	-	-	-	-	+

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Sample number	Age	Gestational age of current pregnancy	No. of pregnancy losses	Gestational age of pregnancy loss(es) (weeks)	High-risk factor identified	HIV status	BV status	BV-associated bacteria		Genital mycoplasmas			
								Concentration of AV	Concentration of GV	MH	MG	UU	UP
B92	34	32	1	16 w	POH, Twin pregnancy	-	-	2.74E+01	-	-	-	-	+
B93	37	28	1	5 w	POH, Hypertension, AMA, Rhesus negative	-	-	-	8.07E-01	-	-	-	+
B94	32	15	4	3 x <15 w; 8 w	POH	-	-	-	-	-	-	-	-
B95	30	28	2	16 w; 16 w	POH, Twin pregnancy	+	+	1.26E+06	8.95E+04	+	-	-	+
B96	32	23	3	12 w; 16 w; 16 w	POH, C/S	-	-	-	-	-	-	-	+
B97	24	21	3	8 w; 12 w; 20 w	POH, Hyperechoic lesions in placenta	-	-	-	5.47E+00	-	-	-	+
B98	22	33	1	20 w	POH, Asthma	-	-	6.14E+00	-	-	-	-	+

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Sample number	Age	Gestational age of current pregnancy	No. of pregnancy losses	Gestational age of pregnancy loss(es) (weeks)	High-risk factor identified	HIV status	BV status	BV-associated bacteria		Genital mycoplasmas			
								Concentration of AV	Concentration of GV	MH	MG	UU	UP
B99	28	25	2	10 w; 20 w	POH, Diabetes, C/S, PET	-	-	1.79E+02	6.16E+01	-	-	-	-
B100	31	20	6	Ectopic (NA); 6 w; 6 w; 6 w; 6 w; 8 w	POH, C/S	-	-	4.24E+01	1.62E+01	-	-	-	+
B101	36	26	1	12 w	POH, C/S, AMA	-	I	5.76E+00	-	-	-	-	-
A10	23	32	2	40 w; 40 w	POH, Hypertension	+	+	7.79E+04	1.69E+05	-	-	-	+
A11	31	26	1	27 w	POH	-	-	7.27E+02	1.87E+03	-	-	-	+
A14	26	21	1	Between 28 w to 36 w	POH	-	I	9.84E+01	-	+	-	-	+
A22	28	37	1	NA	POH, Hypertension	-	-	9.67E+00	-	-	-	-	-
A26	25	16	1	40 w	POH	-	-	6.00E+01	2.40E+04	-	-	+	-
A120	22	37	1	36 w	POH, Hypertension, PET	-	I	4.28E+05	9.74E+05	+	+	-	+

AMA : Advanced maternal age
AV : *Atopobium vaginae*
C/S : Previous caesarean section
GV : *Gardnerella vaginalis*
I : Intermediate
I/C : Inconclusive
IUD : Intrauterine device
MG : *Mycoplasma genitalium*
MH : *Mycoplasma hominis*
NA : Not available/unknown
NVD : Normal vaginal delivery
PET : Pre-eclampsia toxin
POH : Poor obstetric history
UP : *Ureaplasma parvum*
UTI : Urinary tract infection
UU : *Ureaplasma urealyticum*
- : Negative
+ : Positive