Prevalence and characterization of *Gardnerella vaginalis* in pregnant mothers with a history of preterm delivery

By

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Abstract

Risk factors such as intrauterine and vaginal infection put pregnant women at risk for delivering preterm. Bacterial vaginosis (BV) is a polymicrobial clinical syndrome commonly diagnosed in women of reproductive age, with women of African descent with low socioeconomic status and previous preterm delivery at high risk. Although frequently isolated from healthy women, *Gardnerella vaginalis* has been most frequently associated with BV. There is limited data available on the prevalence of BV in Southern Africa; therefore, we embarked on a study to determine the prevalence of BV and *G. vaginalis* in predominantly black communities in the Western Cape, in order to establish the role of *G. vaginalis* in BV.

Women attending various Maternity and Obstetrics units (MOU) in the Cape Peninsula with and without a history of pre-term delivery (PTD) were invited to participate in the study. Several factors were statistically associated with pregnancy history, including location of study population, parity, smoking and presence of clinical symptoms. The presence of *G. vaginalis* was determined by culture in 51.7% of the preterm delivery group (PTDG) and 44% of the full-term delivery group (FTDG) women. BV was detected in 31.13% of PTDG and 23.67% of FTDG by Gram stained analysis according to Nugent scoring criteria, with age and HIV status posing as risk factors. When comparing PTDG and FTDG for an association between the presence of *G. vaginalis* and BV, a stronger association was observed in the PTDG but it was not statistically significant. In both PTDG and FTDG, *G. vaginalis* was isolated significantly more often in women diagnosed with BV at 24.5% ($p < 0.05$). Antibiogram studies revealed both Metronidazole and Clindamycin resistant strains of *G. vaginalis*. *G. vaginalis* Biotype 7 is specifically associated with BV, while Biotype 2 appears to be associated with BV in women with a history of PTD. Accuracy of diagnostic tools were tested and it was determined that Nugent scoring is more sensitive in diagnosing BV (76.04%), but culture for *G. vaginalis* is more specific (83.21%).

Although this study was limited in that we were unable to follow-up pregnancy outcomes, we were able to confirm the perceived role of *G. vaginalis* in BV.

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Declaration

I declare that this work is my own work, that it has not been submitted before for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Megan Stemmet   **DATE**

Signed:……………………..

Chapter 1: Introduction & literature review

1.1. BACKROUND

Infections account for 10-30% of cases of preterm labour (PTL; Dudley, 1997). There are numerous organisms that can cause intrauterine infection, including *Ureaplasma urealyticum, Mycoplasma hominis, Gardnerella vaginalis, Peptostreptococcus* and *Bacteroides* species. Viral infection, although uncommon, may also cause spontaneous preterm delivery (PTD; Goldenberg *et al.*, 2000; Haas *et al.*, 2005) by either haematogenous spread or ascension from the genital tract (Pereira *et al.*, 2005).

There are four stages to ascending intra-uterine infection. The first stage commences when there is a change in vaginal and cervical flora, or presence of cervical pathogens. Bacterial vaginosis (BV) usually manifests in this initial stage. This is succeeded by micro-organisms gaining access to the intrauterine cavity, enabling them to take up residence in the deciduas. The infection may invade foetal vessels or enter the amnion, resulting in microbial invasion of the amniotic cavity or an intra-amniotic infection (stage 3). Finally, bacteria may enter the foetus through various ports, initiating PTL (Romero *et al.*, 2003).

1.2. THE NORMAL VAGINAL ECOSYSTEM

The female genital tract is a complex and dynamic entity that is colonised by an array of different microbiological organisms. The vaginal ecosystem is balanced and controlled by the microbial community that inhabits it. Hormonal and developmental changes may occur due to age, stage of menstrual cycle, pregnancy, method of contraception, frequency of sexual intercourse, number of sexual partners, vaginal douching, use of panty-liners or vaginal deodorants and infection. Similarly, there seems to be differences in the composition of normal vaginal microbiota among different racial/ethnic groups (Larsen & Monif, 2001; Hay, 2002; Zhou *et al.*, 2004; Witkin *et al.*, 2007).

A healthy vaginal ecosystem is dominated by lactic acid and hydrogen peroxide (H_2O_2)-producing *Lactobacillus* species. *Lactobacillus acidophilus* was thought to be the predominant species found in the normal vaginal flora. However, recent studies have shown that *L. crispatus, L. iners, L. gasseri, L. jensenii, L. vaginalis* and *L. gallinarum* may also be present. While the healthy vagina is highly populated by *Lactobacillus* species, several other species, such as *Staphylococcus, Ureaplasma, Gardnerella, Corynebacterium, Streptococcus, Peptrostreptococcus, Bacteroides, Mycoplasma,*

Enterococcus, Escherichia, Veilonella, Bifidobacterium and *Candida*, are present in reduced concentrations (Zhou *et al.*, 2004).

The presence of Lactobacilli helps to preserve a healthy vagina, as it maintains its acidic environment, which acts as a protective mechanism preventing the proliferation of microbial pathogens and the development and transmission of infectious conditions such as sexually transmitted infections (STIs; Pavlova *et al.,* 2002; Wilks *et al.*, 2004; Zhou *et al.*, 2004; Donders, 2007; Witkin *et al.*, 2007; Turovskiy *et al.*, 2011).

Elevated oestrogen levels during puberty results in an increase in glycogen content in vaginal epithelial cells. Glycogen promotes the growth of the beneficial Lactobacilli while simultaneously preventing the growth of potential pathogens. In addition, glycogen metabolism yields lactic acid, promoting a preferred vaginal pH of 3.5-4.5. The moment there is a reduction in Lactobacilli concentration, the vaginal pH increases to approximately 7.0, enabling BV-associated organisms to thrive with a 1000-fold increase in concentrations (Wang, 2000; Hay, 2005; Turovskiy *et al.*, 2011). In cases where Lactobacilli species are unable to thrive, they may be replaced by other lactic acid producing organisms, such as *Atopobium vaginae, Megasphaera* species and *Leptotrichia* species.

1.3.   THE DEFINITION AND AETIOLOGY OF BACTERIAL VAGINOSIS

BV is a clinical syndrome, previously known as non-specific vaginitis that is characterised by a disturbed vaginal ecosystem. The term "non-specific" was chosen to illustrate its poorly understood aetiology, diagnosis and treatment, and the term "vaginitis" used to describe infection of the vulva (Hill, 1985). The identification of additional micro-flora involved and the recognition of a general absence of inflammation, resulted in the term "bacterial vaginosis" being adopted (Hay, 2002; Larsson & Forsum, 2005; Filho *et al.*, 2010; Sobel *et al.*, 2012). Gardner & Dukes (1955) previously described the condition as having a characteristic discharge, increased vaginal pH and microscopically observed squamous vaginal epithelial cells with granular borders, which they named "clue cells" as they gave a clue to its diagnosis (Hill, 1985; Hay, 2002).

BV manifests when the normally high lactobacilli concentrations in the vagina become reduced and are replaced by commensal Gram-negative and anaerobic micro-flora such as *Gardnerella vaginalis, Mobiluncus* species*, Bacteroides* species*, Prevotella* species*, Mycoplasma* species and more recently,

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Atopobium vaginae (Schoonmaker *et al.*, 1991; Spiegel, 1991; Kimberlin & Andrews, 1998; Hay, 2002; Goffinet *et al.*, 2003; Ferris *et al.*, 2004; Livengood, 2009; O'Hanlon *et al.*, 2011). In addition to the organisms previously mentioned, *Peptostreptococcus, Fusobacterium, Veilonella, Eubacterium* species, *Ureaplasma urealyticum* and *Streptococcus viridians* may also be associated with BV (Tabrizi *et al.*, 2006). It has been demonstrated that a symbiotic relationship exists between *G. vaginalis* and *Mycoplasma* to promote the development of BV (Pybus & Onderdonk, 1999).

While healthy individuals without BV have a more homogenous vaginal flora, a key characteristic of BV is its polymicrobial nature. BV-positive patients are rarely, if ever, colonised by a single organism or species (Fredericks & Marrazzo, 2005; Anukam & Reid, 2007). However, which of these organisms is essential for the proliferation and maintenance of BV, remains unclear (Hay, 2002). This review of the literature will focus predominantly on *G. vaginalis* and its association with BV and adverse pregnancy outcomes.

1.4. EPIDEMIOLOGY OF BV AND ITS ASSOCIATION WITH ADVERSE PREGNANCY OUTCOMES

BV is the most common cause of abnormal vaginal discharge affecting 5-51% of women of reproductive age (Schwebke, 2000; Hay, 2002; Bhalla *et al.*, 2007; Koumans *et al.*, 2007; Denny & Culhane, 2009; Verstraelen & Vershelst, 2009; Gillet *et al.*, 2011; Sobel *et al.*, 2012).

There are specific factors that put women at risk of acquiring BV. These include age at first sexual intercourse, change in sexual partners, large number of lifetime sexual partners, coexisting STIs, menses and chronic stress (Gibbs, 2001; Marrazzo, 2011; Turovskiy *et al.*, 2011).

In addition, women who smoke are two times more likely to acquire BV than women who do not. Vaginal douching has also been associated with BV, by assisting in the ascent of lower genital tract organisms. This is a procedure followed by many Afro-Caribbean women, who are also classified as "high risk" for acquiring BV. However whether douching causes BV or whether the presence of BV results in a woman's decision to douche, is unclear (Lliahi-Camp *et al.*, 1996; U.S Preventive Services Task Force, 2008; Wang, 2000). In addition, women from African descent with low socioeconomic

status and previous PTD were found to be predisposed to acquiring BV in a subsequent pregnancy (Elyan and Rund, 2004).

Sexual activity seems to be the most important risk factor, since BV is more commonly diagnosed in sexually active than inactive women (Madhivanan *et al.*, 2008). Although BV is not considered to be a sexually transmitted disease (STD), it has been found in the male sexual partners of women with BV, suggesting it could be sexually associated (Lagacé-Wiens *et al.*, 2008). But BV does not resemble a typical STD as multiple pathogens are involved, most of which normally occur in the vagina of healthy, sexually inactive women. However, more women are diagnosed with BV in STD clinics (33-64%) than prenatal or obstetric clinics (1.7-48.5%; Wang, 2000; Hay, 2005; Mullick *et al.*, 2005; Turovskiy *et al.*, 2011). BV has also been diagnosed in 12-75% of asymptomatic women (Denny & Culhane, 2009: Sobel *et al.*, 2012).

BV has repeatedly been associated with adverse pregnancy outcomes, including PTD, late miscarriage, and premature rupture of membranes (PROM), infection of the chorion, amnion and amniotic fluid, and pelvic inflammatory disease (PID; Kimberlin & Andrews, 1998; Goffinet *et al.*, 2003; US Preventive Services Task Force, 2008; Gondo *et al.*, 2010; Marrazzo, 2011). Furthermore, BV has been detected in 6.8-40% of women admitted for preterm labour at 23-37 weeks (Holst *et al.*, 1994; Goffinet *et al.*, 2003; Kumar *et al.*, 2006). With the aid of Gram stain examination, BV was diagnosed in 11-87% of pregnant women between 23-26 weeks gestation (Hay *et al.*, 1994; Hillier *et al.*, 1995; Govender *et al.*, 1996; Delaney *et al.*, 2001; Goyal *et al.*, 2005). Because the condition may be asymptomatic, BV is diagnosed less frequently (15.8-17%) when using Amsel's criteria (Goyal *et al.*, 2005; Vogel *et al.*, 2006).

The mechanism by which BV-associated organisms cause preterm delivery is uncertain, but it has been linked to reduced leukocyte concentrations, elevated endotoxin levels and protease enzymes that stimulate proinflammatory cytokine and prostaglandin production leading to PTL, and possibly intrauterine death. The secretion of sialidase promotes placental inflammation and weakening of the chorioamniotic membrane, thereby causing lower genital tract organisms to ascend higher into the vagina. The inflammatory response induced by BV, presents paediatric problems as well. Cerebral palsy and bronchopulmonary dysplasia may develop as a result of increased levels of inflammatory cytokines in the amniotic fluid. Additionally, BV serves as a risk factor for long-term neurological complications,

such as hyperactivity and learning disabilities (Nelson *et al.*, 2007; Denny & Culhane, 2009; Gondo *et al.*, 2010; Turovskiy *et al.*, 2011).

BV diagnosed in the first 16 weeks of gestation increases the risk of both late miscarriage and PTD by 1.8-7.3 times, independent of confounding factors. This suggests this is a critical period during gestation that allows BV-associated organisms to gain access to the upper genital tract, setting the stage for subsequent PTD (Kurki *et al.*; 1992; McGregor *et al.*, 1993; Holst *et al.*, 1994; Meis *et al.*, 1995; Gratacós *et al.*, 1998; Denny & Culhane, 2009).

1.5. DIAGNOSTIC PROCEDURES FOR THE IDENTIFICATION OF BV

BV is generally diagnosed by Gram stain examination using Spiegel or Nugent scoring (Spiegel *et al.*, 1983; Krohn *et al.*, 1989; Nugent *et al.*, 1991) and/or clinical symptoms (Amsel *et al.*, 1983), often referred to as Amsel's criteria. The identification and isolation of BV-associated organisms is often used as a screening tool for the diagnosis of BV, either by conventional methods or molecular techniques. Rapid diagnostic methods have also been developed to presumptively identify anaerobic vaginal flora commonly associated with BV, or their by-products.

1.5.1 Amsel's criteria

In 1983, Amsel *et al.* proposed specific criteria for the clinical diagnosis of BV. The criteria involved the identification of three out of four clinical signs and/or symptoms including the identification of a grey, homogenous discharge, a pH greater than 4.5, amine odour in the presence of 10% potassium hydroxide (KOH) and the microscopic observation of clue cells of vaginal epithelial cells. A patient is BV positive if she tests positive for any three of the four criteria.

Amsel's criteria in its entirety is very useful in clinical settings as it does not rely on infrastructural or manual resources (Verstraelen & Verhelst, 2009; Modack *et al.*, 2011). However diagnosis of BV by this method is often erroneous. An accurate diagnosis by Amsel's criteria is dependent on clinical symptoms, but identification of these components is subjective (Chaijareenont *et al.*, 2004; Donders, 2007; Modack *et al.*, 2011).

1.5.1.1 Vaginal Discharge

A thin homogenous discharge may not always be indicative of BV, and in cases of asymptomatic BV in which there is no discharge present, the other three criteria could be analysed to make a diagnosis, but the presence of vaginal discharge has the lowest sensitivity and is non-specific (Verstraelen & Verhelst, 2009; Filho *et al.*, 2010; Mittal *et al.*, 2012).

1.5.1.2   pH and amine production

Vaginal pH >4.5 has good sensitivity for diagnosing BV. Detection of organic acids in vaginal secretions is useful for the presumptive identification of anaerobes in vaginal flora. Previously, gas liquid chromatography (GLC) was used to directly measure the characteristic organic acids in vaginal secretions produced by BV causing bacteria. GLC has since been replaced by electronic sensor array or the electronic nose to test for the presence of trimethylamine, considered to be responsible for the characteristic odour in BV. This can also be tested for with FemExam® test card which combines the detection of amine production with pH testing (Hill, 1985; Hay *et al.,* 2003; West *et al.*, 2003). Another rapid diagnostic method involves the self-test pH glove which allows women to monitor changes in vaginal pH by inserting a glove with a built-in pH indicator into the vagina and subsequently consult their doctor should they observe a pH of 4.7 or more,. This may enable prevention of adverse pregnancy sequelae (Hoyme & Saling, 2004).

Considering the above, the possibility of acknowledging criteria individually or in pairs was investigated. Simoes *et al.* (2006) concluded that any of the Amsel criteria, either individually or in combinations of two, may be equally accurate as three, whereas Mittal *et al.* (2012) believe that using criteria such as pH and amine odour only are insufficient to diagnose BV.

1.5.2   <u>Microscopy</u>

Microscopic examination for the presence of "clue cells" (particularly if ≥20% of vaginal epithelial cells) is considered the most specific predictor of BV (Verstraelen & Verhelst, 2009; Filho *et al.*, 2010; Mittal *et al.*, 2012).

As previously mentioned, microscopically, normal vaginal micro-flora consists largely of *Lactobacillus* morphotypes, while BV is characteristically dominated by *G. vaginalis.* This ignited the development of the Spiegel criteria for quantifying bacterial morphotypes in vaginal smears (Spiegel *et al.*, 1983).

As a result of questionable inter-centre reliability of Spiegel criteria, Nugent *et al.* (1991) set out to develop a scoring system that uses morphotypes that are most reliably identified at different centres. Thus the Nugent score was proposed. Nugent scoring is a modification of the Spiegel criteria that produces a score of 0-10, thus enabling categorisation of the severity of BV. In this system, morphotypes were quantified as follows: 0 equals no morphotypes; 1+ equals ≤1 morphotype; 2+ equals 1-4 morphotypes, 3+ equals 5-30 morphotypes and 4+ equals >30 morphotypes. This system is weighted such that the lack of lactobacilli yields the highest score. As with the Spiegel criteria, lactobacilli and *G. vaginalis* are identified as large Gram-positive bacilli and small Gram-variable bacilli respectively. In addition, bacteria morphologically resembling *Bacteroides* species and *Mobiluncus* species are also identified.

Several researchers have reported on the reliability and reproducibility of this method. It has consistently shown to be convenient, cost effective with high inter-observer and inter-centre reliability, and the scores obtained can give insight into the degree of alteration in vaginal micro-flora (Krohn *et al.*, 1989; Nugent *et al.*, 1991; Platz-Christensen *et al.*, 1995; Chaijareenont *et al.*, 2004; Zarakolu *et al.*, 2004; Goyal *et al.*, 2005; Money, 2005; Libman *et al.*, 2006; Mohanty *et al.*, 2010). Consequently, to maintain its accuracy, care should be taken when preparing and viewing the slides (Joesoef *et al.*, 1991; Larsson *et al.*, 2004; Mohanty *et al.*, 2010). Whether or not it can be used as a screening tool, remains undecided (Krohn *et al.*, 1989; Coppolilo *et al.*, 2003).

While the pathological condition of BV and normal micro-flora is easily defined, some abnormal vaginal micro-flora, such as intermediate flora, cannot be distinctly classified. Intermediate micro-flora are generally characterised by a mixed bacterial flora and is equivalent to a Nugent score of 4-6. Therefore, there is some confusion as to what the intermediate micro-flora represents. It could either represent partial/intermediate BV, or more realistically, it could represent a transient state of BV, which could either regress to full-blown BV or progress to a healthy state (normal micro-flora). Furthermore, intermediate micro-flora has been strongly associated with various pregnancy complications, including mid-trimester pregnancy loss and PTD (Donders *et al.*; 2002; Donders, 2007; Lietich & Kiss, 2007). As a result, a new condition named Aerobic vaginitis (AV) has been described. AV is a condition characterised by the presence of facultative anaerobic micro-organisms such as group-B streptococci and *Escherichia coli*. As with Nugent scoring, AV is diagnosed based on a composite score obtained by

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noting the presence of leucocytes, parabasal cells and background flora. A score of 1 to 2 indicates normal or non-AV flora. A positive AV diagnosis is based on severity, where a score of 3 to 4 indicates slight AV, 5 to 6 indicates moderate AV and a score of greater than 6 is indicative of severe AV (Donders *et al.*, 2002). This classification includes the immune reaction of the host and enables detailed and comprehensive division of micro-flora (Donders, 2007).

1.5.3   Culture

Culture techniques have long been the first approach in identifying the cause of infection. While culture for *G. vaginalis* has a high sensitivity for predicting a clinical diagnosis of BV, Gram staining vaginal smears are more specific. The sole purpose of utilising culture techniques in BV diagnostics would be to identify the organisms involved in the syndrome, but the presence of specific organisms, such as *G. vaginalis*, is not necessarily indicative of BV, as it is recovered in 50-60% of asymptomatic, healthy women. Additionally, several organisms associated with BV are difficult to cultivate, therefore their presence may be missed when using culture techniques (Delaney and Onderdonk, 2001; Kalra *et al.*, 2007; Verstraelen & Verhelst, 2009; Sobel *et al.*, 2012), and thus, it is not recommended as a diagnostic tool for BV.
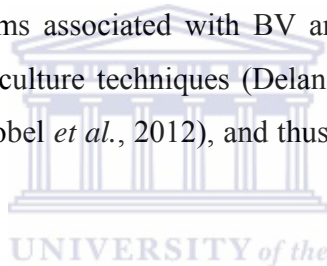
Direct Fluorescent-Antibody developed by Hansen *et al.* (1987) is a simple, rapid test that enables detection of *G. vaginalis* in clinical samples from patients who have been treated with antibiotics and who test negative in culture.

1.5.4   Enzyme Detection

As previously stated, sialidases are important virulence factors of *G. vaginalis*. Sialidase activity can be tested for using BVBlue test which is a simple and rapid diagnostic chromogenic test for detecting BV by changing colour when sialidase activity is detected in vaginal fluid. It was found that 92% of women who tested positive for sialidase had signs of BV. This test would prove to be beneficial in the clinical setting where microscopic analysis is unavailable because it boasts good sensitivity and specificity when compared to Amsel's criteria and Nugent scoring (Myziuk *et al.*, 2003; Bradshaw *et al.*, 2005; Numanović *et al.,* 2008; Verstraelen & Verhelst, 2009).

1.5.5   Molecular biology

Several molecular techniques and rapid diagnostic tests have been developed to aid in diagnosing BV. Since the study done by Burton & Reid (2002) that used Polymerase-chain reaction (PCR)-based techniques to characterise vaginal micro-flora, molecular diagnostic methods have become more popular and increased our understanding of the BV ecosystem and identified new species involved in the syndrome.

The species-specific PCR (sPCR) is a universal technique used to detect a specific microorganism. *G. vaginalis* is detected with 90% accuracy in BV-positive patients with the use of sPCR (Verhelst *et al.*, 2005). Alternatively, Quantitative real-time PCR (QRT-PCR) has excellent sensitivity and specificity, and can thus be used as a tool in diagnosing BV by detecting and quantifying Lactobacilli species, *G. vaginalis* and *Mycoplasma hominis* in the genital tract of women. With this method, *G. vaginalis* was detected significantly more frequently in women clinically diagnosed with BV while the converse occurred with Lactobacilli (Zarifard *et al.*, 2002; Menard *et al.*, 2008).

Multiplex PCR-based methods differentiate BV from non-BV specimens by detecting several bacterial species that are associated with BV. The use of multiplex PCR is a more reliable indicator of BV than detection of a single bacterium, such as *G. vaginalis* (Obata-Yasuoka *et al.*, 2002; Sha *et al.*, 2005). Treating the PCR with proteinase K further enhances its sensitivity for detection of *G. vaginalis*. This test showed 100% correlation with the Nugent scoring system, and can accurately predict BV (Obata-Yasuka *et al.*, 2002). The advantage of PCR- based techniques is that it can be performed on samples directly, without prior culturing and allows identification of microorganisms with fastidious growth requirements. Its only requirement is that the nucleic acids should be intact. Conversely, it is limited by the possibility of false negatives and false positives. False negatives could result from PCR inhibitors present in clinical samples. Examples of PCR inhibitors are blood and mucus that may be present in vaginal secretions, consequently prior DNA extraction is recommended. False positives may occur when broad-range 16s rDNA PCR detect and amplify low concentrations of DNA found in laboratory reagents, DNA extraction kits, and PCR reagents (Fredericks & Marazzo, 2005; Menard *et al.*, 2008); therefore very strict aseptic techniques should be applied.

Several other PCR-based techniques can be used to detect vaginal pathogens, including cloning and denaturing gradient gel electrophoresis of the *16S rRNA* gene or the *chaperonin-60* gene, terminal

restriction fragment length polymorphism (RFLP) of the *16S rRNA* gene and Fluorescence In Situ Hybridization (FISH). By detecting the presence of either *A. vaginae* or *G. vaginalis*, or both, amplification of the chaperonin-60 genesas demonstrated high specificity and sensitivity for the diagnosis of BV using microscopy as a gold standard (Verstraelen & Verhelst, 2009; Dumonceaux *et al.*, 2009; Lamont *et al.*, 2011).

BD diagnostics developed a DNA probe for *G. vaginalis* rRNA that only detects *G. vaginalis* at concentrations $\geq 2 \times 10^5$ CFU/ml. Although this test can be performed in an office setting, it produces better results in a laboratory, and shows promise in detecting *G. vaginalis* in pregnant women as a supplement to Amsel's and Nugent criteria (Briselden & Hillier, 1994).

It is evident that each diagnostic tool has its strengths and weaknesses. It is however essential to assess what needs to be achieved and which specific test will be used. Amsel's criteria and point of care tests are more applicable for clinical settings, while PCR and chromatography can only be utilised in a laboratory. Gram staining appears to be more versatile because slides can be prepared in a clinic and safely transported to the laboratory for staining and analysis.

1.6.   TREATMENT OF BV

The polymicrobial nature of BV makes it a difficult condition to treat (Joesof *et al.*, 1999). BV usually occurs when hormonal changes lower vaginal pH, resulting in alteration of vaginal flora (Curran & Rivlin, 2012). Additionally, BV also has the ability to resolve spontaneously, and this phenomenon occurs in a third of non-pregnant women and half of pregnant women (Nelson & Macones, 2002; Sobel *et al.*, 2012). Considering that the aetiology of the condition remains unexplained, managing it is difficult, resulting in high recurrence rates (Wilson, 2004; Harwich *et al.,* 2010). Treatment of BV is aimed at resolving the symptoms and ensuring that follow-up screening is negative, thus it would not be beneficial to treat asymptomatic patients. Furthermore, treating asymptomatic women could result in symptomatic vaginal yeast infection. However, it is recommended to treat asymptomatic women scheduled for gynaecologic procedures prophylactically (Sobel *et al.*, 2012).

BV is generally treated by either systemic or topical Metronidazole or Clindamycin (Nelson and Macones, 2002). Bacteriotherapy could also be employed, which involves using non-toxic bacteria to dislodge pathogenic organisms (Wilson, 2004). Clindamycin is a bacteriostatic antibiotic that targets

aerobes and Gram-positive and Gram-negative anaerobes, whereas Metronidazole is bactericidal and is usually successful in eliminating Gram-negative anaerobes. Metronidazole does not act against *Lactobacillus* species*;* therefore the natural vaginal ecosystem is not markedly disturbed by the administration of this antibiotic.

Modes of treatment for BV include 500mg oral Metronidazole, twice daily for seven days; 300mg oral Clindamycin twice daily for seven days and 0.75% Metronidazole vaginal gel once or twice a day for 5days (Wang, 2000; Wilson, 2004; Hay, 2005). Alternative treatment options have been suggested, including 250mg oral Metronidazole, 5g Metronidazole intravaginally for 7 days (Curran & Rivlin, 2012), 400mg Metronidazole for 5days (Hay, 2005) and 2% 5g Clindamycin vaginal cream either in a single dose or once daily for 5days (Livengood, 2009).

All modes of treatment demonstrate significant cure rates. Orally administered Metronidazole 500mg twice daily for 7 days demonstrated cure rates of 23-99% whereas slightly lower cure rates of 26-81% were observed for 0.75% Metronidazole vaginal gel (Wang, 2000; Nelson & Macones, 2002; Wilson, 2004; Yudin *et al.*, 2008; Livengood, 2009; Harwich, 2010). Although cure rates of 70-94% were demonstrated for oral Clindamycin 300mg twice daily for 7 days (Nelson & Macones, 2002; Yudin *et al.*, 2008), the cure rate for topical Clindamycin is debatable. Livengood (2009) reported reduced cure rates (30-37%) for 2% Clindamycin vaginal cream, compared to a report by Wang (2000), indicating cure rates of 70-90% (Marrazzo, 2011). Despite the prosperity of treatment, 25-30% of women will experience recurrence within 1-3 months and 50% at 6-12 months. Recurrence could have two possible causes; 1) re-infection with a biotype different from the original infecting biotype, or 2) inadequate eradication of infecting organisms, and thus inability of normal protective lactobacilli to re-establish (Anukam *et al.*, 2006; Numanović *et al.*, 2008; Sobel *et al.*, 2012).

Since the first report on the use of yogurt to successfully treat BV during pregnancy (Neri *et al.*, 1993), several researchers have tested the efficacy of Lactobacilliary probiotics for the treatment of BV (Shalev, 2002; Vitali et al., 2012). Probiotics are live strains of micro-organisms with beneficial effects on a patient's health when administered in adequate amounts. Probiotics can be useful in the treatment of BV not only by replacing reduced lactobacilli concentrations but also by stimulating an

immunomodulation process. A 40% cure rate has been demonstrated in the use of probiotics to treat BV (Krauss-Silva *et al.*, 2011; Othman, 2012).

1.7.   EVIDENCE FOR THE ROLE OF G. VAGINALIS IN THE PATHOGENESIS OF BV

In order for micro-organisms to successfully colonise the host and avoid elimination by host defences, they require enzymes capable of penetrating the mucosal barrier. The pathogenesis of BV is complex and relatively unknown, but it is apparent that factors that enable invasion and colonisation of vaginal epithelia play an important role (Catlin, 1992; Diejamaoh *et al.*, 1999).

Biofilm production involves a group of bacteria adhering to the epithelial surface of a cell (Wilson, 2001). Several studies have demonstrated the presence of a biofilm in patients with infections such as periodontal disease, endocarditis, foreign-body related infections, as well as BV. Biofilm formation has been shown to be an important virulence factor in BV, because its presence enables an increase in antibiotic and host immune defence resistance, as micro-organisms are able to become more concentrated than is possible in vaginal fluid (Hale *et al.*, 2006). Primarily, BV was thought to be caused solely by *G. vaginalis*. Although *G. vaginalis* is isolated in 95% of cases of BV and encompasses 90% of the polymicrobial BV biofilm (Hale *et al.*, 2006), it is also found in healthy women, and pure cultures of *G. vaginalis* do not cause BV, resulting in its pathogenic potential being questioned. However, it continues to play a pivotal role in development of BV (Turovskiy *et al.*, 2011).

In the initial stages in the pathogenesis of BV, *G. vaginalis* forms "clue cells"; this indicates its adherence ability. As a result of this ability, it is capable of forming a biofilm and is thus considered an initial coloniser. An initial coloniser is an organism that is able to adhere to the cell surface and subsequently enables the attachment of other micro-organisms with low innate pathogenic potential, to thrive in the vagina (Kalinka *et al.*, 2004). *G. vaginalis* also produces sialidase and mucinase, which aids in attachment and invasion of the upper genital tract, and plays a role in bacterial nutrition, cellular interactions, immune response evasion and pathogenicity of bacteria (Briselden *et al.*, 1992; Wiggins *et al.*, 2001; Cauci *et al.*, 2003; Bradshaw *et al.*, 2005; Santiago *et al.*, 2011). An additional, equally important, virulence factor of *G. vaginalis* is the production of a protein toxin, vaginolysin. Vaginolyin is a pore-forming toxin that disturbs the plasma membrane, subsequently causing cell lysis (Pleckaityte *et al.*, 2011).

Cumulatively, the virulence properties of *G. vaginalis* enables adherence to vaginal epithelium, biofilm production and secretion of vaginolysin leading to cell lysis and tissue destruction. The biofilm produced by *G. vaginalis* is extremely resistant to H_2O_2 and lactic acid (Patterson *et al.*, 2010). This further enhances the proliferation and attachment of BV-associated organisms to the biofilm, even in the presence of lactobacilli (Udayalaxmi *et al.*, 2011). This suggests that other BV-associated organisms take advantage of *G. vaginalis'* powerful virulence properties and depend on *G. vaginalis* to prosper. Depending on growth conditions, *G. vaginalis* also produces synergistic or antagonistic compounds against lactobacilli and other vaginal bacterial compounds (Teixeira *et al.*, 2010). It is been consistently illustrated that BV pathogens occur in various combinations, but always together with *G. vaginalis*, suggesting a symbiotic relationship exists between *G. vaginalis* and other BV pathogens (Pybus & Onderdonk, 1999; Menard *et al.*, 2008).

Biotyping and starch hydrolysis was initially introduced as a means of identifying and differentiating *G. vaginalis* from other coryneforms of vaginal origin (Piot *et al.*, 1982). The technique was modified from tests used to identify *Campylobacter fetus* (Harvey, 1980) and Group B Streptococci (Hwang and Ederer, 1975), and was standardized and optimised according to *G. vaginalis'* reaction. Generally, *G. vaginalis* can be characterised into specific biotypes based on biochemical reactions such as lipase and β-galactosidase activity, and hippurate hydrolysis (Numanović *et al.*, 2008).

Hippurate is a salt/ester of hippuric acid. Many bacterial species are able to hydrolyse hippurate and this ability is often tested to characterise and identify microorganisms (Kodaka *et al.*, 1982). Hippurate is hydrolysed in a multi-step process, with the first by-product being benzoic acid. Initially hippurate hydrolysis was tested using ferric chloride as an indicator to detect benzoic acid. However, rapid tests have been developed to detect glycine, which is the second by-product, by using ninhydrine as an indicator to detect glycine production (Hwang and Ederer, 1975; Harvey, 1980; Luechtefeld and Wang, 1982; Piot, 1982). Currently, there are several lipase assay methods that enable detection of lipase activity by making use of colorimetric or fluorescent substrates to be detected via spectrophotometry. A bacterium's ability to synthesize and secrete lipase is influenced by environmental factors, such as ions, carbon sources and presence of non-metabolizable polysaccharides (Kurooka and Kitamura, 1978; Jeager *et al.*, 1994). Lipases produced by bacteria hydrolyse esters of glycerol on a glycerol backbone of lipid substrates. This reaction takes place when hydrophobic lipid substrates are added to hydrophilic

aqueous solution. When there is a sudden increase in lipase activity and the substrate forms an emulsion, an interfacial area is created. This is called interfacial activation, which is characteristic of lipases. β-Galactosidase is an enzyme that breaks down lactose into glucose and galactose, which are then used as carbon sources. It is coded for by lacZ gene in the lac operon of *Escherichia coli*. This is an example of a reporter gene, in which a virulence gene is fused with a structural gene. The synthetic chromogenic compound, o-nitrophenyl-β-D-galactoside (ONPG), is used as a substrate in place of lactose. When ONPG is cleaved, galactose and o-nitrophenol is produced. While ONPG is colourless, o-nitrophenol has a yellow colour. The rate of production of o-nitrophenol is proportional to the concentration of β-galactosidase. Therefore, the production of yellow colour can be used to determine β-galactosidase concentration (Miller, 1972; Salyers and Whitt, 1994).

Currently, two biotyping schemes exist. The scheme by Piot *et al.* (1984) recognises eight biotypes, while Benito *et al.* (1986) identified seventeen. The distribution of biotypes of *G. vaginalis* differs among geographical regions (Piot *et al.*, 1984). Piot *et al.* found that while biotypes 1, 2 and 5 were more frequently isolated in Seattle, USA and Antwerp, Belgium, biotypes 1 and 5 are most common in Nairobi, Kenya. In another study testing subjects from Seattle, biotypes 1, 5 and 6 were found to be most common (Briselden and Hillier, 1990). In Bosnia, Numanvovic *et al.* (2008) found biotypes 2, 3, and 7 occurred most frequently.

Certain biotypes have also been associated with BV. While Piot *et al.* found no particular biotype associated with BV, other studies found biotypes 1, 2, 3, 4, 5 and 7 to be associated with BV. Biotypes 1, 2, 4, 5 and 7 are most prevalent in women presenting with clinical symptoms of BV (Benito *et al.*, 1986; Ison *et al.*, 1987; Scott *et al.*, 1987). The male sexual partner of women diagnosed with BV harboured the same biotypes, supporting the notion that *G. vaginalis* is sexually transmitted. However, this does not mean that BV is a STD; instead *G. vaginalis* is a pre-cursor for the development of BV (Numanvović *et al.*, 2008).

1.8. SUMMARY AND OBJECTIVES

Bacterial vaginosis (BV) is characterised by reduced Lactobacilli species concentration and an overpopulation of Gram-negative anaerobic bacteria, including *Gardnerella vaginalis*, *Mobiluncus*, *Bacteriodes*, *Atopobium vaginae*, *Prevotella* and *Mycoplasma* species. BV is a polymicrobial and very complex vaginal syndrome, the aetiology of which is relatively unknown. It affects 50-80% of the female population of reproductive age.

G. vaginalis seems to play an important role in the development of BV, as several of its virulence factors are required to allow attachment of other BV-associated pathogens and the production of BV symptoms. Additionally, a symbiotic relationship has been observed between *G. vaginalis* and several other organisms involved in BV, suggesting a significant role for *G. vaginalis* in the establishment of the BV biofilm and therefore, for preterm birth.

Null hypothesis (H_0)

H_o: *G. vaginalis* is not associated with BV in mothers with a history of preterm birth.

The aim of this study is to establish the prevalence of *G. vaginalis* in pregnant women with a history of preterm delivery.

The objectives are:
- To investigate maternal risk factors for previous pregnancy outcome and BV
- To examine for the prevalence of *G. vaginalis* in BV patients
- To determine the accuracy of culture and microscopy for detecting *G. vaginalis* in BV
- To characterise the *G. vaginalis* isolates for their biotypes and antibiograms

Chapter 2: Materials and methods

2.1.   SUBJECT SELECTION

A total of 301 pregnant women receiving antenatal care at four Midwife and Obstetrics Units, including Mowbray, Khayelitsha, Mitchell's Plain and Gugulethu were selected to participate in this study. Of these, 150 had a history of full-term delivery (FTD) with no pre-term delivery and 151 had a history of preterm delivery (PTD). Women were enrolled in the study on their first prenatal visit. Informed consent was obtained by means of a consent form after they received detailed information regarding the nature of the study and sampling methods to be used. The research assistant at the clinic completed a short questionnaire outlining demography, medical history and social behaviour, and for purposes of anonymity, patients were identified by a sample number, which was recorded along with their folder numbers (Appendix A). Where necessary, an interpreter was employed. Specific inclusion criteria were implemented for enrolment in the study. For the pre-term delivery group (PTDG), subjects were selected based on the following inclusion and exclusion criteria: pregnant woman at ≥28weeks gestation with a history of at least one previous spontaneous pre-term delivery (between 24 and 37weeks gestation). For the full-term delivery group (FTDG) selection was determined by the patient having at least one previous, uncomplicated spontaneous term delivery (after 37weeks gestation) with no pre-term delivery. Patients not falling within the above-mentioned gestational period and patients, who had received antibiotic therapy three weeks prior to being examined, were excluded from the study.

Ninety (29.90%) samples were collected from Mowbray Maternity hospital (MMH), 74 (24.58%) from Khayelitsha MOU (KMOU), 86 (28.57%) from Mitchell's Plain MOU (MPP) and 51 (16.94%) from Gugulethu MOU (GUG).

2.2.   SAMPLE COLLECTION

Vaginal samples were collected without a speculum using sterile cotton swabs and immediately transferred to Amies transport media with charcoal (18114CST, Sterilin) and transported to the laboratory.

Amsel criteria were not used for diagnostic purposes since the aim of the study was to test the accuracy of the use of microscopy and culture only for the detection of BV and *G. vaginalis*.

2.3. LABORATORY/MICROSCOPICAL EXAMINATION

Vaginal smears were prepared from the swab for microscopic examination. The smears were allowed to air-dry before being heat-fixed and Gram stained by Koppeloff's modification using safranin as counter stain. Using the Nugent scoring system of bacterial morphotypes (Nugent *et al.*, 1991), the presence or absence of BV was recorded.

In conjunction with Nugent scoring, slides were also examined for clue cells. An epithelial cell was considered a clue cell if the borders of the cell were completely surrounded with bacteria and had edges that look "grainy". If any part of the border of the cell was clear, it was not considered a clue cell. More importantly, a sample was only positively diagnosed with clue cells if there were ≥20% clue cells per field.

2.3.1. <u>Nugent Score</u>

The Nugent score is a scoring system from 0-10, which enables gradation of the severity of BV. The scoring criterion computes a weighted quantification to yield a score of 0 – 10. At least four microscopic fields were examined for approximately two minutes per field. A Nugent score was allocated based on the average of the four fields examined.

Three morphotypes (*Lactobacillus*, *Bacteroides*/*Gardnerella* & *Mobiluncus* spp.) were quantitated on a scale of 1 to 4 as follows: 1+ (< 1 cell per field), 2+ (1 to 5 cells per field), 3+ (6 to 30 cells per field) and 4+ (> 30 cells per field). The total scores were then computed by adding the weighted quantitation (0 to 4+) of the three morphotypes to yield a score of 0-10. A score of 1-3 was representative of a vaginal ecosystem dominated by *Lactobacillus* species and was thus categorised as normal flora. A score of score 7-10 was allocated when lactobacilli were severely reduced and replaced with *Gardnerella/Bacteriodes*-like morphotypes, thus indicatingbacterial vaginosis. Where a score of 4-6 was allocated, indicating intermediate flora, a mixed vaginal ecosystem was observed, whereby both *Lactobacillus* and *Gardnerella/Bacteriodes*-like morphotypes were present (Table 1).

Table 1: Scoring vaginal Gram stains for Bacterial Vaginosis (Nugent *et al.*, 1991)

Morphotypes	Number per oil immersion field	Score
Lactobacillus-like (parallel-sided, Gram-positive rods)	> 30	0
	5-30	1
	1-4	2
	<1	3
Gardnerella/Bacteriodes-like (tiny, Gram-variable coccobacilli and rounded, pleomorphic, Gram-negative rods)	<1	1
	1-4	2
	5-30	3
	>30	4
Curved Gram-variable rods	0	0
	1-2	1
	3-4	2

2.3.2. Aerobic vaginitis

Gram stained slides were examined to classify cases of intermediate flora as possible aerobic vaginitis (AV), as described by Donders *et al.* (2002). Briefly, Lactobacillary grades formed the basis of a combined score (Table 2). Lactobacillary grades (LBG) are defined as follows: I = numerous pleomorphic lactobacilli, no other bacteria; IIa = mixed flora, but predominantly lactobacilli; IIb = mixed flora, but proportion of lactobacilli severely decreased due to increased number of other bacteria: III = lactobacilli severely depressed or absent because of overgrowth of other bacteria. One or more of the following were added to the above LBG: leucocytes, presence of toxic leucocytes, presence of parabasal cells (PBCs), no PBCs: score = 0; PBCs representing <10% of epithelial cells: score = 1; PBCs >10%: score = 2 and background flora. For background flora, a score = 0 indicated unremarkable flora, or debris and bare nuclei from lysed epithelial cells; score = 1 indicated course or small bacilli resembling lactobacilli morphotypes; score = 2 indicated prominent or chained cocci. A composite score of 1-2 signifies normality, while a score of 3-4 indicates slight AV and score of 5-6 corresponded to moderate AV. A score of greater than 6 (but not more than 10) represented severe AV (Table 2).

Table 2: Criteria for the microscopic diagnosis of aerobic vaginitis

AV Score	LBG	No. of leukocytes	Proportion of toxic leukocytes	Background flora	Proportion of PBC
0	I and IIa	≤10/ hpf	None or sporadic	Unremarkable or cytolysis	None or < 1%
1	IIb	>10/hpf and ≤ 10/epithelial cell	≤ 50% of leukocytes	Small coliform bacilli	≤ 10%
2	III	> 10/epithelial cell	> 50% of leukocytes	Cocci or chains	>10%

Hpf: high power field (100 × magnification).

2.4. CULTURE

Samples were cultured on Columbia blood agar base (CM0331B, Oxoid) containing *G. vaginalis* selective supplements (SR0119E, Oxoid) and 5% sheep blood and streaked for single colonies. Cultures were incubated in an anaerobic jar containing gas-generating kit (BR0038B, Oxoid) at 37^0C for 48hours. After incubation, cultures were examined for small, grey, opaque β-haemolytic colonies. Smears were made for Gram staining to confirm the presence of *G. vaginalis*. The presence of small Gram-variable pleomorphic bacilli was considered to be indicative of *G. vaginalis*.

Samples were stored in Prolab Microbank cryovials (PL170M, Davies Diagnostics) at -80^0C for all subsequent tests.

2.5. ANTIBIOTIC SENSITIVITY TESTS

Antibiotic sensitivity tests were performed on all isolates of *G. vaginalis*. Isolates were sub-cultured on *G. vaginalis* selective agar and incubated anaerobically, to ensure purity of cultures. A bacterial suspension was created in 5ml Todd Hewitt Broth (CM0189, Oxoid) to a concentration of 6-9 x 10^8 colony-forming units (CFUs) per millilitre. Tubes were briefly vortexed, after which 100µl of broth suspension was inoculated onto each plate. The test was done in duplicate for accuracy. The inoculum was then evenly spread over the plate surface using a sterile glass spreader. Plates were allowed to dry very briefly before placing antibiotic discs, 100µg Sulphonamide, 50µg Metronidazole and 10µg Clindamycin (DD0011, DD0008 and CT0015, Oxoid), in three corners of the plate. Culture plates were

incubated anaerobically at 37^0C and examined at 24 and 48 hour intervals to observe sensitivity or resistance. If sensitivity was observed, the diameter of the zone of inhibition was measured using a ruler. Zone diameter size was measured as the distance from the antibiotic disc to the edge of the area of bacterial growth and interpreted as per manufacturer's instructions as follows:

Clindamycin: resistant $= \leq 14$mm; sensitive $= \geq 21$mm

Metronidazole: resistant $= \leq 10$mm; sensitive $= \geq 10$mm

2.6. *G. VAGINALIS* BIOTYPING

Several biochemical tests were performed to characterise *G. vaginalis* into 8 biotypes (Piot *et al.*, 1982). All samples were sub-cultured prior to testing to ensure samples were viable and pure.

2.6.1. Lipase activity assay

Lipase activity was measured using QuantiChrom^{TM} Lipase assay kit (BioAssay Systems, DLPS-100). It is a rapid colorimetric determination of lipase activity at 412nm. The assay was performed as per manufacturer's instructions.

Working agent was prepared by mixing colour reagent with assay buffer, and then adding the dimercaptopropanol tributyrate (BALB) reagent. As this assay is based on a kinetic reaction, addition of the working reagent should be swift.

Samples were dispersed in phosphate buffered saline and centrifugation at 14,000rpm for 5min. In a 96 well microtitre plate, 150μl sterile distilled water was added to the first well and 150μl calibrator was added to the second well. Ten microliters of sample was added to subsequent wells, after which 140μl working reagent was added. A spectrophotometry reading of the plate was taken at OD_{412nm} on a plate reader (Anthos 2010) after 10min (OD_{10min}) and 20min (OD_{20min}) incubation periods at room temperature. Lipase activity was calculated using the formula below:

$$\text{Activity} = \frac{OD_{20min} - OD_{10min}}{OD_{Calibrator} - OD_{H2O}} \times 735 \text{ (U/L)}$$

where OD_{20min} and OD_{10min} are the OD_{412nm} values of the sample at 20 min and 10 min, respectively. $OD_{Calibrator}$ and OD_{H2O} are the OD_{412nm} values of the Calibrator and water at 20 min. The number "735" is the equivalent activity (U/L) of the calibrator under the assay conditions.

2.6.2.   β-Galactosidase activity

A substrate solution was prepared, consisting of 0.4% 2-nitrophenyl-β-D-galactopyronoside (48712-1GM, Merck & Co), 75ml sterile distilled water and 25ml buffer solution ($NaH_2PO_4.1H_2O$ + 40ml dH_2O (pH7)). The substrate solution was dispensed in 1ml microtubes in 0.5ml aliquots. Microtubes were inoculated with a loopful (~10μl) of 24hour culture of *G. vaginalis* and incubated at 37^0C in a water bath for 4hours and 18hours. Tubes were examined after every incubation period for the appearance of a yellow colour (Piot *et al.,* 1984)

2.6.3.   Hippurate Hydrolysis assay

A rapid hippurate test kit (Hardy Diagnostics, Z52) was used to determine the ability of *G, vaginalis* isolates to hydrolyse the compound hippurate. The test was performed as per manufacturer's instructions. Briefly, a hippurate test tube containing 20mg Sodium Hippurate was dissolved by adding 3-4 drops (~0.2ml) distilled water to the tube. The tube was then heavily inoculated with cultures using a sterile inoculating loop. Tubes were then incubated for 2 hours at 35-37°C. Meanwhile, the Ninhydrin indicator solution was reconstituted in 2ml distilled water. The solution was mixed by shaking the dropper bottle vigorously, and allowed to dissolve at room temperature until tubes were removed from the incubator. Following the incubation period, 2 drops of Ninhydrin indicator solution was added to the hippurate-sample mixture. Tubes were subsequently re-incubated at 35-37°C and observed at 10 minute intervals for 30min for a deep blue/purple colour change, indicating a positive test.

Following completion of the above tests, samples were categorised according the scheme tabulated in Table 3.

Table 3: Biotypes of *G.vaginalis*

Feature	Biotype number							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
B-Galactosidase	+	-	-	+	-	+	-	+
Lipase	+	+	+	+	-	-	-	-
Hippurate Hydrolysis	+	+	-	-	+	+	-	-

Piot et al., 1984

2.7. STATISTICAL ANALYSIS

Statistical analyses were carried out using SPSS 17.0 (SPSS Inc., Chicago, IL). Categorical data were analysed using the chi-square test. Where an expected cell value was greater than 20%, Fisher's exact test was used. Non-parametric data was analysed using the Mann-Whitney *U*-test. A *p*-value of 0.05 was considered as significant. Logistic regression was used to determine significant correlations.

The accuracy of the diagnostic tests (microscopy and culture) was expressed by using the following formula:

Sensitivity: TP/ (TP+ FN), Specificity: TN/ (FP + TN), positive predictive value (PPV): TP/ (TP + FP), negative predictive value (NPV): TN/ (FN + TN), Positive odds ratios: Sensitivity/1 − specificity while negative odds ratios were obtained by the following formula: FN/TP + FN divided by TN/FP +TN. The percentage accuracy for each method was calculated using the formula: TP + TN/sample number X 100. TP: True positive, TN: true negative, FN: false negative, FP: false positive.

Chapter 3: Results

3.1. DEMOGRAPHICS

Three-hundred and one women with a history of either pre-term (PT) or full-term (FT) delivery participated in this study. Prior to swab sample collection, participants were asked to complete a questionnaire in order to identify factors which might pose a health risk. The responses to the questionnaire are summarized in Table 4 and Table 5. Logistic regression analysis demonstrated a good model of fit and 35% of the independent variables reflected in this study explained the variance in pre-term delivery group (PTDG). The variables STI, HIV, clinical symptoms and douching had to be extracted from the model as collectively, they had too many missing data. Except for location and smoking, none of the variables had a significant effect on pregnancy outcomes.

3.1.1. Location of population groups

The four locations from which samples were collected serve a mainly under-privileged, black and coloured community. Most of the study patients attended Mowbray (MMH; 29.9%), and a statistically significant association between location and pregnancy outcomes was observed ($p = 0.000$). The women attending Gugulethu (GUG) and Khayelitsha (KMOU) Maternity units are predominantly black, while MMH and Mitchell's Plain (MPP) serve both black and coloured patients. Women from GUG were found to have a 14 times greater chance of having a history of PTD than those from MMH (Table 4).

3.1.1. Age

The age of the study population ranged from 19-42 years with an average age of 28 years. In PTDG and full-term delivery group (FTDG), most of the study patients were in the 26-30 year age range viz. 52 (34.4 %) for PTDG and 54 (36%) for FTDG (Table 4). Forty-four (29.1 %) of PTDG were in the age range of 19-25 years, while 36 (24%) of FTDG were in this range. The lowest percentage of women participating were in the ≥41year age range, 2 (1.3%) in PTDG and 6 (4%) in FTDG. There was no statistically significant association between age of subject and history of pregnancy outcomes.

Table 4: Summary of population demographics

Characteristic	Frequency n (%)			Significance levels
	PTDG n=151	FTDG n=150	Total n=301	
Locations [a]				
• MMH	56 (37.1)	34 (22.7)	90 (29.9)	
• KMOU	26 (17.2)	48 (32.0)	74 (24.6)	$p = 0.000$
• MMP	34 (22.5)	52 (34.7)	86 (28.6)	
• GUG	35 (23.2)	16 (10.7)	51 (16.9)	
Age Distribution				
• 19-25 Years	44 (29.1)	36 (24.0)	80 (26.6)	
• 26-30 Years	52 (34.4)	54 (36.0)	106 (35.2)	
• 31-35 Years	34 (22.5)	38 (25.3)	72 (23.9)	$p = 0.730$
• 36-40 Years	14 (9.3)	13 (10.7)	27 (9.0)	
• $\geq$ 41 Years	2 (1.3)	6 (4.0)	8 (2.7)	
Marital Status				
• Single	72 (47.7)	73 (48.7)	145 (48.2)	
• Married	70 (46.4)	64 (42.7)	134 (44.5)	$p = 0.846$
• Boyfriend	8 (5.3)	6 (4.0)	14 (4.7)	
Gravidity				
• 0-3	118 (78.1)	128 (85.3)	246 (81.7)	
• 4-5	31 (20.5)	22 (14.7)	53 (17.6)	$p = 0.695$
• $\geq$7	2 (1.3)	0 (0.0)	2 (0.7)	
Parity				
• 0-3	146 (96.7)	144 (96.0)	290 (96.3)	
• 4-5	5 (3.3)	6 (4.0)	11 (3.7)	$p = 0.003$
• $\geq$7	0 (0.0)	0 (0.0)	0 (0.0)	

Level of Education				
• Primary school	6 (4.0)	8 (5.3)	14 (4.7)	
• Secondary school	138 (91.4)	135 (90.0)	273 (90.7)	*p* = 0.681
• Tertiary/University	5 (3.3)	5 (3.3)	10 (3.3)	
Employment				
• Yes	59 (39.1)	59 (39.3)	118 (39.2)	
• No	91 (60.3)	89 (59.3)	180 (59.8)	*p* = 1.000
• Unknown	1 (0.66)	2 (1.3)	3 (0.9)	

a: MMH, Mowbray MOU; KMOU, Khayelitsha MOU; MMP, Mitchell's Plain MOU; GUG Gugulethu MOU

3.1.2. Marital Status

While there was no statistically significant difference (*p* = 0.846) between marital status across the two groups (PTDG and FTDG), it should be noted that the number of single and married women only differed by a small margin, 145 (48.2%) and 134 (44.5%) respectively (Table 4).

3.1.3. Parity & Gravidity

The total number of previous pregnancies averaged at 2.75 (±1.10) For PTDG, it ranged between 1 and 7 with an average of 2.78 (±1.22), while for FTDG the range was between 2 and 6 with an average of 2.65 (± 0.96). In both PTDG and FTDG, majority of the women had 0-3 previous pregnancies (78.1% and 85.3% respectively). Similarly, a parity of 0-3 was most prevalent in both FTDG and PTDG. While a statistically significant difference of average parity was demonstrated (*p* = 0.003), none was detected with gravidity across PTDG and FTDG (Table 4).

3.1.4. Education

All participants obtained some level of education (Table 4). A large percentage (90.7%) reached secondary school, 138 (91.4%) of which were in PTDG and 135 (90%) in FTDG, but only 62 (41.1%) and 55 (36.4%) respectively completed secondary school. From each group, 5 (3.3%) received tertiary education.

3.1.5. Employment

More than half of the study population was unemployed and an enough an equal number (59, 39.1% and 39.6% respectively) of patients from both groups were employed. However, those who were employed held low income occupations such as municipal workers, cashiers or domestic workers (Table 4).

3.2. HEALTH ASSESSMENT

3.2.1. Smoking & Alcohol consumption

A statistically significant relationship was observed between smoking ($p = 0.020$) but not alcohol consumption ($p = 0.413$) and a history of PTD. There were more smokers among PTDG (18.54%) than among FTDG (8.61%; Table 5). However, the majority were non-smokers. Similar results were seen with alcohol consumption. Twelve of 151 (7.95%) patients in PTDG consumed alcohol, while 139 (92.05) did not. Likewise, 17 of 150 (11.26%) of FTDG consumed alcohol and 132 (87.42%) did not. Logistic regression revealed non-smokers were 83% less likely to have a history of PTDG.

3.2.2. History of STI

Overall, a larger percentage (72.43%) of the study group had not been previously diagnosed with a sexually transmitted infection (STI; Table 5). Although 21.19% of PTDG were previously diagnosed with an STI compared with 12.67% of FTDG, this difference was not statistically significant ($p = 0.350$).

3.2.3. HIV status

Although not all patients disclosed their HIV status, 25.83% of PTDG and 23.18% of FTDG were HIV-positive (Table 5), and 7.5% PTDG and 10.6% FTDG had not been previously tested for HIV. Of the study population, 66.23% of the PTDG and 65.56% of the FTDG were HIV-negative.

3.2.1. Clinical symptoms

Clinical symptoms which were apparent upon sample collection included urinary tract infections and vaginal discharge. In PTDG, only 3.02% and12.67% of FTDG presented with urinary symptoms (Table 5). Although, there were more women with no clinical symptoms in PTDG (40.40%) than FTDG (9.33%), vaginal discharge was more common in FTDG (78%) than in PTDG (59.95%).

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Table 5: Summary of Health assessment information obtained from questionnaire

Characteristic	Frequency (%)			
	PTDG n=151	FTDG n=150	Total n=301	Significance level
Cigarette Smoking				
• Yes	28 (18.54)	13 (8.61)	41(13.62)	$p = 0.020$
• No	123 (81.46)	137 (91.33)	260 (86.38)	
Alcohol consumption				
• Yes	12 (7.95)	17 (11.26)	29 (9.63)	
• No	139 (92.05)	132 (87.42)	271 (90)	$p = 0.413$
• Unknown		1 (0.33)	1 (0.33)	
STI				
• Yes	32 (21.19)	19 (12.67)	51 (16.95)	
• No	102 (67.55)	116 (77.33)	218 (72.43)	$p = 0.350$
• Unknown	17 (11.26)	15 (10)	32 (10.63)	
HIV				
• Negative	100 (66.23)	99 (65.56)	199 (66.11)	
• Positive	39 (25.83)	35 (23.18)	74 (24.58)	$p = 0.982$
• Unknown/non reactive	12 (7.95)	16 (10.60)	28 (9.30)	
Clinical Symptoms				
• Vaginal discharge	86 (59.95)	117 (78)	176 (58.47)	
• Urinary symptoms	2 (3.02)	19 (12.67)	21 (6.98)	$p = 0.009$
• None	61 (40.40)	14 (9.33)	59 (19.60)	
Personal Hygiene Bathing option				
• Bath	39 (25.83)	32 (21.33)	71 (23.59)	
• Shower	12 (7.95)	7 (4.67)	19 (6.31)	$p = 0.265$
• Hand wash	100 (66.23)	107 (71.33)	207 (68.77)	
Douching				
• Yes	0 (0)	1 (0.67)	1 (0.33)	
• No	39 (25.83)	44 (29.33)	83 (27.57)	$p = 1.000$
• Unknown	69 (45.70)	105 (70)	174 (57.81)	

3.2.1. Personal hygiene practices

As a result of poor living conditions, most of the women involved in this study (207, 68.77%), had to resort to washing in a basin as a bathing option. A smaller number of patients kept clean by showering, i.e. 7.95% of PTDG and 4.67% of FTDG, while the rest, 25.83% and 21.33% respectively, chose to bath (Table 5).

Only one person, from FTDG, practiced douching, while 175 women did not respond to that question for reasons which remain unknown. There was no statistically significant relationship between the PTDG and FTDG for personal hygiene practices and a previous PTD (Table 9).
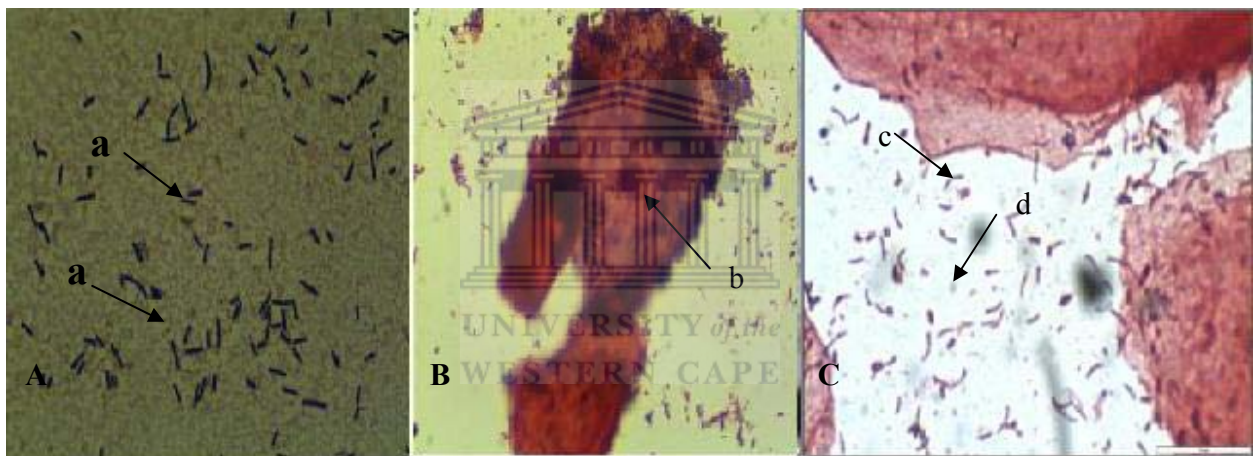
Figure 1: Photographic representation of Gram stain of vaginal smears from (A) a patient harbouring high number of Lactobacilli ssp.(a) indicative of NF, (B) clue cell, the borders of which are obscured by bacteria, a characteristic feature of BV and (C) a patient with high levels of curved Gram negative rods (c) and Gram-variable rods (d) indicating BV.

3.3. PREVALENCE OF BACTERIAL VAGINOSIS ASSESSED MICROSCOPICALLY

3.3.1 Microscopic evaluation

The Amsel's criteria are generally used to make a diagnosis of BV within a clinical setting. The presence of clue cells is probably the most important criterion and can be detected microscopically either by saline wet mount or, less often, by Gram-staining. However, the clinics at which samples were collected are severely understaffed and can get quite busy. As a result, only vaginal swab samples could be collected, and therefore all clinical criteria to diagnose BV could not be applied. However, we used this limitation to establish the accuracy of microscopy and culture to detect BV in mothers associated with previous PTD.

Vaginal swabs were smeared on a glass slide and Gram-stained using safrinin as counterstain. Slides were analysed and scored according to Nugent scoring criteria (Nugent *et al.*, 1991) as described in Chapter 2 (Materials & Methods). Briefly, bacterial morphotypes with a score of 0-3 represents high numbers of Lactobacilli spp., indicative of normal vaginal micro-flora, while a score of 4-6 indicates intermediate flora and a score of 7-10 represents high numbers of Gram-negative to Gram-variable rods and absence of lactobacilli, which is indicative of bacterial vaginosis. Gram-stained slides were examined for the presence of clue cells, which are epithelial cells whose borders are completely surrounded by bacteria. More importantly, a sample was only positively diagnosed with clue cells if there were ≥20% clue cells per high-power field (Figure 1).

In PTDG, a total of 151 slides were examined, while 150 slides were examined in FTDG. Gram stain analysis of vaginal swabs revealed that 96 of 301 (32%) of the study population harboured vaginal flora indicative of BV, 47 of which were in PTDG and 49 were in FTDG (Table 6).

While no statistically significant association ($p = 0.618$) was observed between a history of PTD and the presence of clue cells, clue cells were more frequently detected in PTDG (13.9%) women than women from FTDG (11.3%; Table 6), particularly in patients with a positive BV diagnosis, demonstrating a statistically significant association between BV and clue cells ($p = 0.000$). The majority of the population had a score indicative of normal micro-flora, with similar numbers being observed in PTDG (52.3%) and FTDG (52%). A small group of women presented with intermediate flora. Intermediate

flora was observed in 16.6% of PTDG while 14.7% of FTDG had intermediate flora (Table 6). No statistically significant association was found between a history of PTD and a positive diagnosis of BV ($p = 0.893$).

With the application of reclassification of intermediate flora to Aerobic vaginitis (AV; Donders *et al.et al..*, 2002) AV was diagnosed in 42 (27.8%) of PTDG and 37 (24.7%) of FTDG subjects (Table 6). Twenty-eight of the 47 (59.6%) subjects previously classified as intermediate was reclassified as AV, of which 13 (27.7%) were from PTDG and 15 (31.9%) from FTDG.

Table 6: Microscopy

	PTDG $_{n=151}$ (%)	**FTDG $_{n=150}$ (%)**	**Significance levels**
NF	79 (52.3)	78 (52.0)	
IF	25 (16.56)	22 (14.7)	
BV	47 (31.1)	49 (32.7)	0.893
Clue cells	21 (13.9)	17 (11.3)	0.618
AV	42 (27.8)	37 (24.7)	0.649

NF: Normal flora; IF: intermediate flora; BV: Bacterial vaginosis; AV: Aerobic vaginitis

3.4. PREVALENCE OF *GARDNERELLA VAGINALIS*

3.4.1. Isolation

The presence of *G. vaginalis* was determined by culture and confirmed by Gram staining (Table 7). *G. vaginalis* was detected in 144 (47.8%) of the 301 (95% confidence interval (CI) 0.42-0.54) women studied of which 78 (51.7%; CI 0.42-0.54) had a previous PTD and 66 (44%; 95% CI 1.191-2.001) had a previous FTD, with no significant relationship ($p = 0.225$) between the isolation of *G. vaginalis* and a previous PTD being observed (Figure 2).
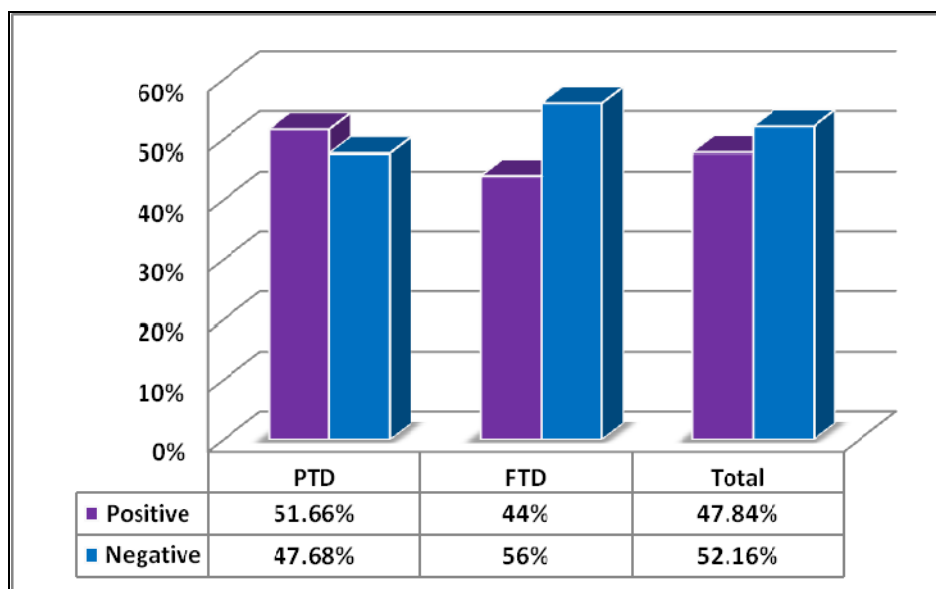
Figure 2: Graphical presentation indicating the percentage of women who harboured *Gardnerella vaginalis* in both PTDG and FTDG

3.4.2. Antibiograms

Clinical isolates of *G. vaginalis* were tested for their susceptibility to antibiotics generally used to treat BV (Table 7). The sample numbers for PTDG and FTDG were reduced to 73 and 60 respectively as a result of plate contamination and subsequent loss of samples. Antimicrobial activity towards Clindamycin was similar between the two groups. Clindamycin exhibited antimicrobial activity towards 75.3% of PTDG and 76.7% of FTDG isolates. Antimicrobial patterns remained constant over the 48 hour incubation period (Table 7).

As indicated in Figure 3 and Figure 4, all *G. vaginalis* isolates in PTDG were resistant to Metronidazole, and with the exception of 1 (1.7%) isolate from FTDG, so were the isolates from FTDG. Sulphonamide was used as a reference as *G. vaginalis* is known to be resistant to its action.

Table 7: Prevalence of *G. vaginalis*

	Isolation	Susceptible strains	
		Metronidazole	**Clindamycin**
PTDG	51.7%	0.0%	75.3%
FTDG	44.0%	1.7%	76.7%

	Resistant	Sensitive
■ Metronidazole	100%	0%
■ Sulphonamide	100%	0%
■ Clindamycin	24.70%	75.30%
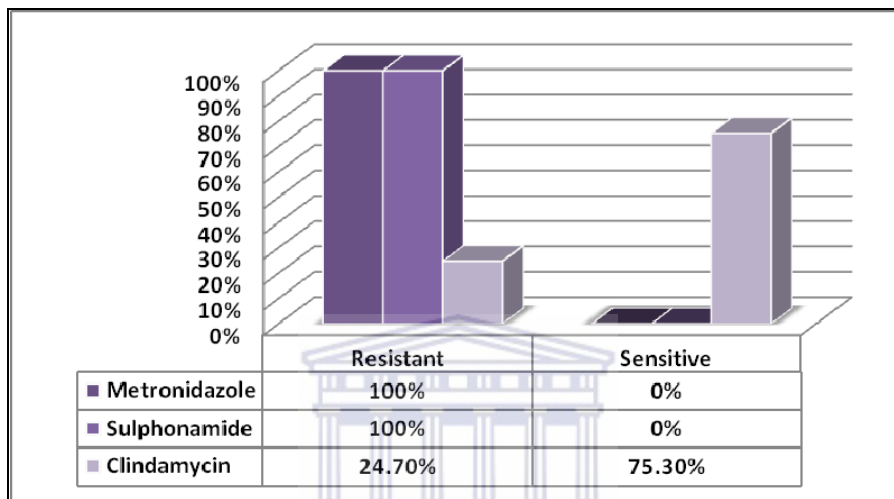
Figure 3: Antimicrobial activity against clinical isolates of *Gardnerella vaginalis* collected from women with a history if Preterm Delivery

	Resistant	Sensitive
■ Metronidazole	98.30%	1.70%
■ Sulphonamide	100%	0%
■ Clindamycin	23.30%	76.70%
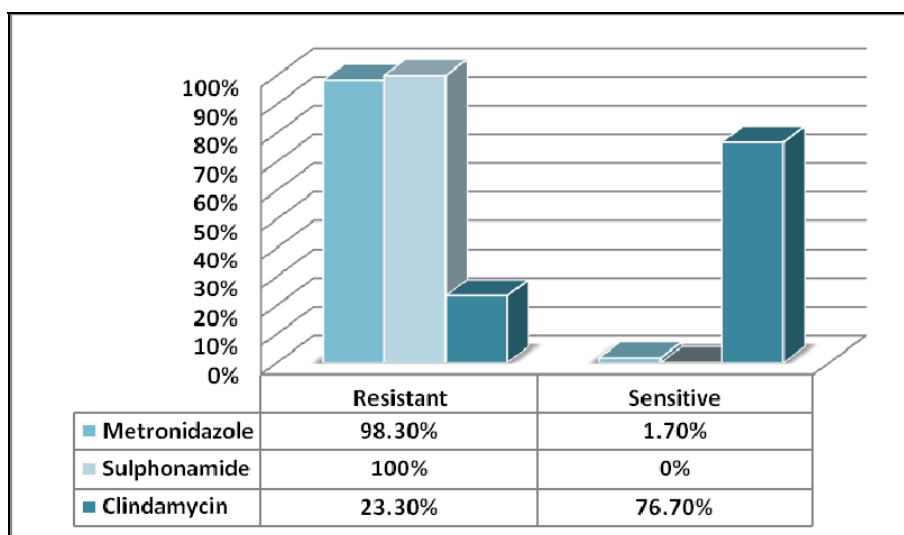
Figure 4: Antimicrobial activity against clinical isolates of *Gardnerella vaginalis* collected from women with a history if Full term Delivery

3.4.3. Frequency of Biotypes

Gardnerella vaginalis isolates were characterised into eight biotypes using the biochemical tests for lipase and β-galactosidase activity and hippurate hydrolysis. Table 8 illustrates the varying frequencies at which biotypes were detected. In PTDG biotype 5 occurred most frequently at 24.7%, followed by biotype 7 at 23.3% and biotype 2 at 17.8%. Biotypes 1 and 4 occurred less frequently at 4.1% each. A significant association was observed between previous PTD and FTD for biotypes ($p = 0.003$), with biotypes 2, 4 and particularly 5, showing the most significant difference between PTD and FTD (Table 8). Similarly; in FTDG certain biotypes prevailed. However, here biotypes 3 and 4 occurred most frequently at 20% each, with biotype 4 demonstrating a significant difference ($p = 0.017$), while biotype 2 was detected the least frequently at 5%. Interestingly to note biotypes 2, 3 and 4 are characterised by lipase and β-galactosidase activity, while 5 and 7 are characterised as having negative reactions for these tests.

Table 8: Frequency of biotypes in PTDG and FTDG

	Biotype (%)								Significance level
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	
PTDG (n=73)	3 (4.1)	13 (17.8)	5 (6.8)	3 (4.1)	18 (24.7)	7 (9.6)	17 (23.3)	7 (9.5)	$p = 0.003$
FTDG (n=60)	5 (8.3)	3 (5.0)	12 (20.0)	12 (20.0)	5 (8.3)	4 (6.7)	10 (16.7)	9 (15.0)	
Significance levels		$p = 0.011$	$p = 0.079$	$p = 0.017$	$p = 0.005$				

3.4.4. Metronidazole and Clindamycin resistant biotypes

Of all the isolates Biotype 7 was the most resistant to Metronidazole (27/133, 20.3%), however biotype 5 was more resistant in PTDG (24.7%) and biotypes 3 and 4 (20.0%) in FTDG. The one isolate that was sensitive to Metronidazole, from FTDG, was characterised as biotype 5. Within FTDG, no statistically significant association between biotypes and Metronidazole resistance was observed ($p = 0.283$; Figure 5).
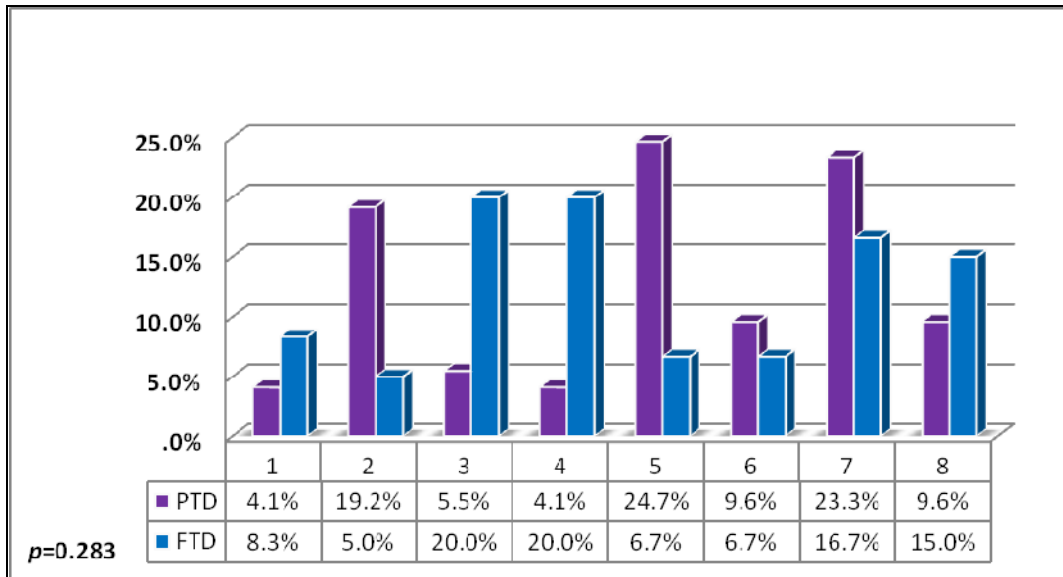
Figure 5: Metronidazole resistance associated with biotypes in FTD and PTD

Considering that Clindamycin resistance was low, in PTDG biotype 5 had the highest resistance to Clindamycin (9.6%), whereas biotype 4 was the most resistant strain in FTDG.

In both PTDG and FTDG, no statisically significant association between Clindamycin resistance and biotypes was observed (Figure 6).
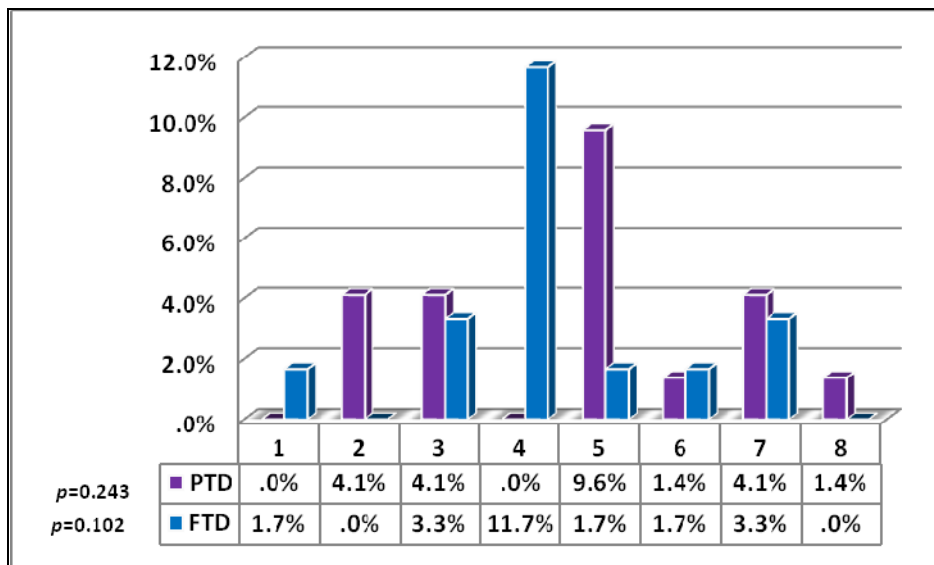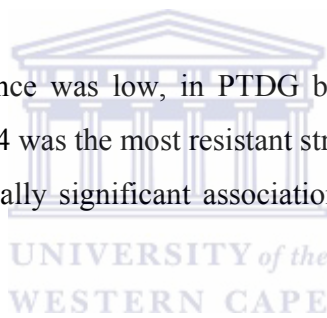
Figure 6: Clindamycin resistance associated with biotypes in FTD and PTD

3.5.   RISK FACTORS

The prevalence BV, AV, clue cells, *G. vaginalis* and Metronidazole and Clindamycin resistant strains were compared to various demographic and clinical data to determine which confounding factors serves as risk factors for the above conditions.

3.5.1   Location of population groups

BV was common in women attending MMH, with the highest percentage observed in FTDG (22.7%). In PTDG AV was diagnosed most frequently in subjects attending MMH, while the highest frequency of AV in FTDG was observed at KMOU (Table 9). The highest prevalence of *G. vaginalis* In PTDG was observed at MMH (35.9%), while for FTDG the prevalence of *G. vaginalis* was highest at KMOU (37.9%).

Although no statistically significant association was observed between location of populations where sample collection took place and Metronidazole resistance, the highest percentage of resistant strains were isolated from 37% of PTDG subjects at MMH, and 40.7% of FTDG at KMOU. Similar results were observed with Clindamycin resistance (Table 9).

3.5.2   Age

BV, *G. vaginalis* and Metronidazole resistant strains was more prevalent in the19-25year age group (Table 9) with BV diagnosed in 12.0% in FTDG and in 11.9% of PTDG; *G. vaginalis* was isolated in 66.66% of FTDG subjects and 69.23% of PTDG, no statistically significant association was observed between groups.

In PTDG, AV was most prevalent in the 26-30 year age range (10.6%), whereas it was more prevalent in the 19-25 years age range in FTDG (8.7%; Table 9).

G. vaginalis was rarely detected in the ≥41 year age group (1.3% of PTDG and 1.5% of FTDG). Clindamycin resistant strains were more common in the 26-30 year age groups

3.5.3   Marital status

In both groups, BV and AV (13.9% PTDG and 14.7% FTDG) was more prevalent in single women, but there was no statistically significant association between relationship status and BV (*p* = 0.288; Table 9). In FTDG, *G. vaginalis* was detected more from women who were unmarried (53.0%) than married (31.8%) or in a relationship (boyfriend; 4.5%). Whereas in

PTDG, there was a small difference in the isolation of *G. vaginalis* in those who were single (47.4%) and those who were married (48.7%), but this was not of statistical significance (Table 9).

In PTDG, Metronidazole and Clindamycin resistance was highest in those who were married, whereas in FTDG resistance was highest in single women.

3.5.4 Education

Both BV and *G. vaginalis* was more frequently detected in women with secondary school education, but no statistical significant association was observed between BV and education level (Table 9).

3.5.5 Smoking & Alcohol consumption

In PTDG, BV was diagnosed in 42 (27.8%) of non-smokers and 45 (29.8%) of non-drinkers. In FTDG BV was diagnosed in 46 (30.9%) of non-smokers and 43 (29.1%) of non-drinkers in the FTDG (Table 9).

Almost equal numbers of *G. vaginalis*-positive women, 84.6% of PTDG and 84.4% of FTDG, were non-smokers, while fewer *G. vaginalis*-positive women from FTDG (10.61%) smoked compared to 15.4% of PTDG women. *G. vaginalis* was isolated in only 5.1% of PTDG and 15.2% of FTDG subjects who consumed alcohol (Table 9).

3.5.6 History STI & HIV status

For both PTDG and FTDG subjects, the prevalence of BV was highest in women who were not previously diagnosed with an STI, 23.2% and 23.3% respectively. The prevalence of BV was significantly low in HIV-positive subjects ($p = 0.004$; Table 9).

A small percentage of *G. vaginalis*-positive women were previously diagnosed with an STI, but the percentage of PTDG (15.4%) was higher than FTDG (12.1%).

G. vaginalis was detected in 28.2% of HIV-positive women in PTDG and in 27.3% of HIV-positive women in FTDG (Table 9).

3.5.7 Clinical symptoms

BV prevalence was much greater in subjects presenting with vaginal discharge than urinary symptoms (Table 9).

In PTDG, majority of women who presented with vaginal discharge (64.1%) harboured *G. vaginalis* and this was less than those in FTDG (72.7%). In addition, more FTDG subjects with *G. vaginalis* colonization (15.2%) presented with urinary symptoms than PTDG (1.3%). *G. vaginalis* was isolated from more healthy women in the PTDG (29.49%) than FTDG (12.1%).

In both PTDG and FTDG, Metronidazole resistance was high in those presenting with urinary symptoms, whereas Clindamycin resistance was highest in those with vaginal discharge (Table 9).

3.5.8 Personal Hygiene

BV was frequently detected in women making use of hand washing in a wash basin, which is not an adequate cleansing method. Similarly, *G. vaginalis* was isolated mostly in women who washed in a washing basin, 74.4% in PTDG and 71.2% in FTDG. While smaller percentages showered and bathed (Table 9). Metronidazole and Clindamycin resistance was highest in those making use of hand-washing in a wash basin as a bathing option (Table 9).

None of the women diagnosed with BV or from whom *G. vaginalis* was isolated practiced douching.

Table 9: Risk factors for BV and demographic and clinical data

	Significance levels	PTDG				Antibiograms		FTDG				Antibiograms	
		BV n=151	Clue cells n=151	AV n=151	GV n=78	Metro. n=73	Clinda. n=18	BV n=150	Clue cells n=150	AV n=150	GV n=66	Met. n=59	Clin. n=14
Locations													
• MMH	p = 0.000	18 (11.9)	11 (7.3)	20 (13.2)	28 (35.9)	27 (37.0)	6 (33.3)	34 (22.7)	3 (2.0)	6 (4.0)	14 (21.2)	14 (23.7)	1 (7.1)
• KMOU		8 (5.3)	2 (1.3)	6 (4.0)	14 (17.9)	13 (17.8)	2 (11.1)	17 (11.3)	6 (4.0)	14 (9.3)	25 (37.9)	24 (40.7)	6 (42.9)
• MPP		6 (4.0)	1 (0.7)	9 (6.0)	19 (24.4)	18 (24.7)	4 (22.2)	15 (10.0)	4 (2.7)	12 (8.0)	19 (28.8)	15 (25.4)	5 (35.7)
• GUG		15 (9.9)	7 (4.6)	7 (4.6)	17 (21.8)	15 (20.5)	6 (33.3)	8 (5.3)	4 (2.7)	5 (3.3)	8 (12.1)	6 (10.2)	2 (14.3)
Age Distribution													
• 19-25 Years	p = 0.785	18 (11.9)	7 (4.6)	13 (8.6)	26 (33.3)	24 (32.9)	7 (38.9)	18 (12.0)	3 (2.0)	13 (8.7)	20 (30.3)	20 (33.9)	5 (35.7)
• 26-30 Years		15 (9.9)	7 (4.6)	16 (10.6)	28 (35.9)	27 (37.0)	7 (38.9)	17 (11.3)	9 (6.0)	10 (6.7)	23 (34.8)	19 (32.2)	6 (42.9)
• 31-35 Years		11 (7.3)	5 (3.3)	8 (5.3)	17 (21.8)	17 (23.3)	3 (16.7)	12 (8.0)	3 (2.0)	8 (5.3)	16 (24.2)	13 (22.0)	3 (21.4)
• 36-40 Years		2 (1.3)	1 (0.7)	2 (1.3)	6 (7.7)	5 (6.8)	1 (5.6)	0 (0.0)	1 (0.7)	5 (3.3)	4 (6.1)	5 (8.5)	0 (0.0)
• ≥41 Years		0 (0.0)	0 (0.0)	2 (1.3)	1 (1.3)	0 (0.0)	0 (0.0)	1 (0.7)	1 (0.7)	0 (0.0)	1 (1.5)	0 (0.0)	0 (0.0)
Marital Status													
• Single	p = 0.244	25 (16.6)	9 (6.0)	21 (13.9)	37 (47.4)	33 (45.2)	6 (33.3)	27 (18.0)	8 (5.3)	22 (14.7)	35 (53.0)	32 (54.2)	8 (57.1)
• Married		21 (13.9)	12 (7.9)	19 (12.6)	38 (48.7)	3 (4.1)	1 (5.6)	17 (11.3)	7 (4.7)	10 (6.7)	21 (31.8)	2 (3.4)	0 (0.0)
• Boyfriend		1 (0.7)	0 (0.0)	2 (1.3)	3 (3.8)	37 (50.7)	11 (61.1)	2 (1.3)	1 (0.7)	2 (1.3)	3 (4.5)	18 (30.5)	6 (42.9)
Level of Education													
• Primary school	p = 0.092	3 (2.0)	1 (0.7)	2 (1.3)	3 (3.8)	1 (1.4)	0 (0.0)	2 (1.3)	0 (0.0)	2 (1.3)	5 (7.6)	5 (8.5)	1 (7.1)
• Secondary school		42 (27.8)	19 (12.6)	38 (25.2)	72 (92.3)	68 (93.2)	17 (94.4)	47 (31.3)	17 (11.3)	33 (22.0)	58 (87.9)	51 (86.4)	13 (92.9)
• Tertiary/University		1 (0.7)	1 (0.7)	1 (0.7)	2 (2.6)	3 (4.1)	1 (5.6)	0 (0.0)	0 (0.0)	2 (1.3)	3 (4.5)	3 (5.1)	6 (42.9)

	p-value												
Employment													
• Yes	p = 0.527	17 (11.3)	8 (5.3)	15 (9.9)	27 (34.6)	25 (34.2)	6 (33.3)	13 (8.7)	8 (5.3)	13 (8.7)	26 (39.4)	23 (39.0)	7 (50.0)
• No		30 (20.0)	13 (8.6)	27 (17.9)	50 (64.1)	47 (64.4)	12 (66.7)	36 (24.0)	9 (6.0)	24 (16.0)	40 (60.6)	36 (61.0)	7 (50.0)
Cigarette Smoking													
• Yes	p = 0.007	5 (3.3)	3 (2.0)	10 (6.6)	12 (15.4)	11 (15.1)	3 (16.7)	3 (2.0)	0 (0.0)	1 (0.7)	7 (10.6)	7 (11.9)	0 (0.0)
• No		42 (27.8)	18 (12.0)	32 (21.2)	66 (84.6)	62 (84.9)	15 (83.3)	46 (30.7)	17 (11.3)	36 (24.0)	59 (89.4)	52 (88.1)	14 (100.0)
Alcohol consumption													
• Yes	p = 0.319	2 (1.3)	1 (0.7)	4 (2.6)	4 (5.1)	4 (5.5)	1 (5.6)	6 (4.0)	2 (1.3)	4 (2.7)	10 (15.2)	8 (13.6)	0 (0.0)
• No		45 (29.8)	20 (13.2)	38 (25.2)	74 (94.9)	69 (94.5)	17 (94.4)	43 (28.7)	15 (10.0)	32 (21.3)	55 (83.3)	50 (84.7)	14 (100.0)
STI[Φ]													
• Yes		8 (5.3)	7 (4.6)	7 (4.6)	12 (15.4)	12 (16.4)	4 (22.2)	9 (6.0)	5 (3.3)	4 (2.7)	8 (12.1)	7 (11.9)	3 (21.4)
• No		35 (23.2)	12 (7.9)	30 (19.9)	55 (70.5)	50 (68.5)	10 (55.6)	35 (23.3)	11 (7.3)	27 (18.0)	52 (78.8)	46 (78.0)	10 (71.4)
HIV[Φ]													
• Positive		19 (12.6)	9 (6.0)	15 (9.9)	22 (28.2)	21 (28.8)	4 (22.2)	13 (8.7	3 (2.0)	12 (8.0)	18 (27.3)	16 (27.1)	4 (28.6)
• Negative		23 (15.2)	9 (6.0)	24 (15.9)	49 (62.8)	48 (65.8)	14 (77.8)	32 (21.3)	13 (8.7)	20 (13.3)	42 (63.6)	38 (64.4)	9 (64.3)
Clinical Symptoms													
• Vaginal discharge	p = 0.657	27 (17.9)	1 (0.7)	2 (1.3)	50 (64.1)	1 (1.4)	10 (55.6)	38 (25.3)	2 (1.3)	2 (1.3)	48 (72.7)	9 (15.3)	13 (92.9)
• Urinary symptoms		1 (0.7)	12 (7.9)	23 (15.2)	1 (1.3)	45 (61.6)	0 (0.0)	7 (4.7)	14 (9.3)	33 (22.0)	10 (15.2)	42 (71.2)	0 (0.0)
Personal Hygiene													
Bathing option													
• Hand wash	p = 0.244	38 (25.2)	17 (11.3)	31 (20.5)	58 (74.4)	54 (74.0)	13 (72.2)	36 (24.0)	14 (9.3)	27 (18.0)	47 (71.2)	41 (69.5)	10 (71.4)
• Bath		6 (4.0)	3 (2.0)	10 (6.6)	15 (19.2)	14 (19.2)	4 (22.2)	11 (7.3)	3 (2.0)	9 (6.0)	12 (18.2)	11 (18.6)	3 (21.4)
• Shower		3 (2.0)	1 (0.7)	1 (0.7)	5 (6.4)	5 (6.8)	1 (5.6)	1 (0.7)	0 (0.0)	1 (0.7)	5 (7.6)	5 (8.5)	1 (7.1)
Douching[Φ]													
• Yes		0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
• No		13 (8.6)	6 (4.0)	12 (7.9)	16 (20.5)	16 (21.9)	0 (0.0)	16 (10.6)	4 (2.7)	8 (5.3)	25 (37.9)	23 (39.0)	0 (0.0)

BV: Bacterial vaginosis; AV: Aerobic vaginitis, GV: *G. vaginalis*, Metro: Metronidazole, Clind: Clindamycin

Φ: unable to calculate significant levels due to abundance of missing data

## 3.6. ASSOCIATION OF MICROSCOPY WITH PREVALENCE OF *GARDNERELLA VAGINALIS* & BIOTYPES

3.6.1. *G. vaginalis* isolates

A cross-examination was done to determine how many women diagnosis with BV were colonised with *G. vaginalis*, and whether there was a difference between PTDG and FTDG (Figure 7).

Among FTDG, more than a third (39.3%) of the women who were not colonised by *G. vaginalis*, presented with normal vaginal flora, while this was seen in only a small number (12.7%) of *G. vaginalis* colonizers. On the other hand, the *G. vaginalis*-positive women were more prone to having vaginal flora indicative of bacterial vaginosis (22.7%) and fewer *G. vaginalis*-negative women had abnormal vaginal flora. Low numbers of both *G. vaginalis*-positive (8.7%) and *G. vaginalis*-negative (6%) presented with intermediate flora.

Similar results were seen among PTDG. *G. vaginalis*-negative women had predominantly normal flora (36.4%) and while there were more *G. vaginalis*-negative women presenting with intermediate flora (6.62%, 10/151), a small number (5.3%) had flora representative of BV. In addition, BV was more prevalent among *G. vaginalis*-positive women (25.8%) when compared to normal flora (15.9%) in PTDG (Figure 7).
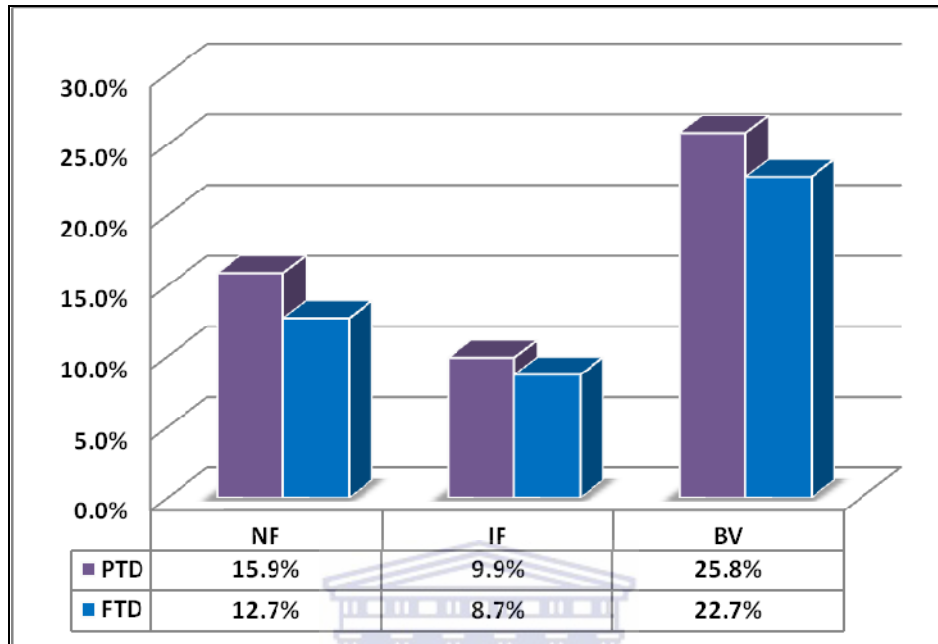
Figure 7: Prevalence of *Gardnerella vaginalis* compared with Microscopy

NF = normal flora, IF = intermediate flora, BV = bacterial vaginosis; PTDG = preterm birth (n=151), FTDG = normal birth (n=150)

3.6.2. Biotypes associated with BV and AV

As demonstrated in Table 10, biotypes were cross referenced with the microscopic examinations for AV and BV.

In PTDG, a statistically significant association was observed between a history of PTD and BV ($p = 0.000$) and AV ($p = 0.000$). Biotype 2 was the dominant biotype in patients with BV (19.2%), whereas biotype 8 prevailed in patients with AV (16.7 %; Figure 8).

However in FTDG, biotype 4 dominated both in patients with BV (18.4%) and AV (10.8%; Figure 8), but this association was only significant with BV ($p = 0.000$; Table 10).

Table 10: Association between microscopy suggestive of BV and AV, and biotypes

	PTDG		FTDG	
	BV $_{n=47}$	AV $_{n=42}$	BV $_{n=49}$	AV $_{n=37}$
1	2 (4.3)	1 (2.4)	2 (4.1)	2 (5.4)
2	9 (19.2)	6 (14.3)	3 (6.1)	1 (2.7)
3	3 (6.4)	2 (4.8)	3 (6.1)	3 (8.1)
4	1 (2.1)	0 (0.0)	9 (18.4)	4 (10.8)
5	7 (14.9)	6 (14.3)	4 (8.2)	2 (5.4)
6	5 (10.6)	4 (9.5)	2 (4.1)	2 (5.4)
7	7 (14.9)	6 (14.3)	7 (14.3)	3 (8.1)
8	3 (6.4)	7 (16.7)	4 (8.2)	3 (8.1)
Significance level	***p* = 0.000**	***p* = 0.000**	***p* = 0.000**	***p* = 0.618**
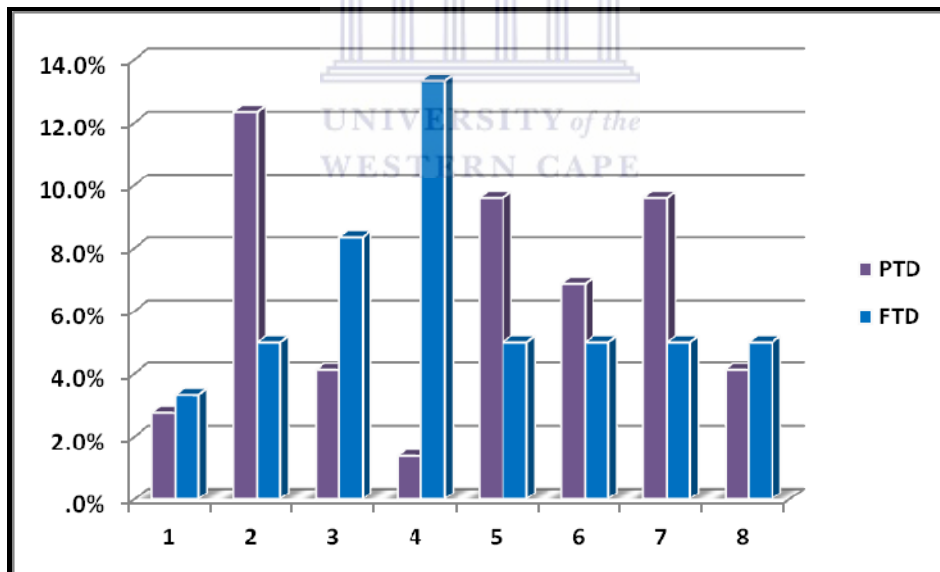
Figure 8: Cross-analysis of biotypes with BV observed in patients with history of preterm delivery

3.6.3. Antibiograms associated with BV

Metronidazole and Clindamycin resistant strains were cross-referenced with Nugent scoring results to determine if antibiotic resistance is associated with BV. For both PTDG and FTDG, Metronidazole resistance was demonstrated in approximately 50% of subjects diagnosed with BV (37 of 73 and 29 of

60 respectively). Metronidazole resistance was observed in subjects with normal vaginal micro-flora, but to a lesser degree (31.5%; Figure 9).

	PTD (n=73)	FTD (n=60)
BV	50.7%	48.3%
IF	17.8%	21.7%
NF	31.5%	28.3%
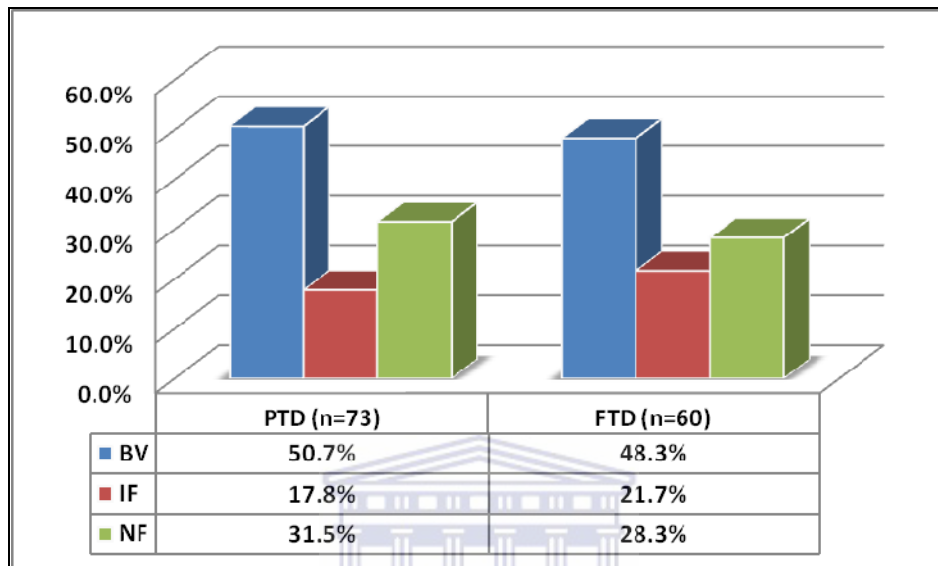
Figure 9: Association of metronidazole resistant strains of *G. vaginalis* in patients with BV

Taking into account that fewer subjects demonstrated resistance to Clindamycin, naturally Clindamycin resistance would be low among BV-positive subjects (11.0% in PTDG and 13.3% in FTDG; Figure 10), and no statistically significant association between BV and neither Metronidazole nor Clindamycin resistance was observed.
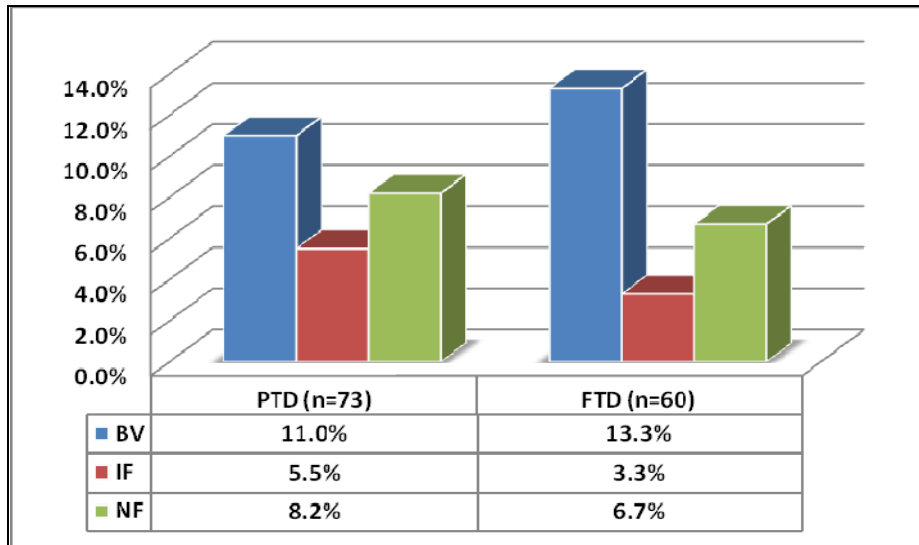
Figure 10: Association of clindamycin resistant strains of *G. vaginalis* in patients with BV

3.7.   ACCURACY OF MICROSCOPY AND *G. VAGINALIS* FOR DETECTING BV

The Nugent scoring system is often used to diagnose BV in a research or clinical laboratory. Furthermore, the detection of *G. vaginalis* from patients suspected of having BV is often used as a predictor of preterm delivery. However, *G. vaginalis* can also be isolated from healthy patients; therefore the accuracy of *G. vaginalis* detection as an indicator of BV was investigated.

When Nugent scoring was used as the reference standard, the PPV for isolating *G. vaginalis* in culture was 84.7% with a sensitivity of 76.04%. The specificity (87.3%) and PPV (82.98) of the Nugent score was highest in mothers with a history of PTD compared with FTD mothers with an accuracy of 62% (Table 11).

Table 11: Comparison of Nugent scoring with culture

Test	Sens.	Spec.	PPV	NPV	Acc	ODDS+	ODDS-
Culture	76.04	72.6	84.68	83.21	22	3.13	0.2
PTDG	61.90	87.30	82.98	69.62	62.25	-25.4	0.4
FTDG	69.39	75.64	64.15	79.73	62	-6.25	0.1127

Sens.: sensitivity, Spec.: specificity, Acc.: Percentage accuracy, PPV: positive predictive value, NPV: negative predictive value, Odds +: positive odds ratio,

Odds -: negative odds ratio.

When culture was used as the reference standard, the sensitivity for a positive Nugent score dropped to 62.93% while the specificity increased 83.2%. Unlike the Nugent score, culture was not able to differentiate between PTDG and FTDG mothers (Table 12).

Table 12: Comparison of Culture for *Gardnerella* with microscopy

Test	Sens.	Spec.	PPV	NPV	Acc	ODDS+	ODDS-
Nugent	62.93	83.21	76.04	72.61	22	-20.28	0.37
PTDG	69.39	71.08	61.9	87.30	31.33	-1.69	0.143
FTDG	64.15	79.73	69.39	75.650	21.38	-15.58	0.31

Sens.: sensitivity, Spec.: specificity, Acc.: Percentage accuracy, PPV: positive predictive value, NPV: negative predictive value, Odds +: positive odd ratio,

Odds -: negative odd ratio.

Chapter 4: Discussion & Conclusion

Bacterial vaginosis (BV) has been shown to be a risk factor in adverse pregnancy outcomes (Holst *et al.et al..,* 1994; Hillier *et al.*, 1995; Lliahi-Camp *et al.,* 1996). Previous studies (Krohn *et al.*, 1989; Kimberlin & Andrews, 1998) have demonstrated that *Gardnerella vaginalis* plays a major role in the development of BV. Thus, we embarked on a study to establish the prevalence of BV and *G. vaginalis* in pregnant women with and without a history of preterm delivery, in order to establish an association of BV and *G. vaginalis* with adverse pregnancy outcomes. In addition, *G. vaginalis* was characterised by biotyping to establish which biotypes are most prevalent in this Western Cape population.

4.1. RISK FACTORS FOR BV

Adverse pregnancy outcomes, such as preterm delivery (PTD), have become a rare event in industrialised countries, but remain a serious problem in the developing world. In 2005 the highest rates of preterm birth reported were in Africa (11.9%), while the lowest were in Europe (6.2%). Not only does PTD impose a risk of maternal and/or neonatal mortality, but it may also cause severe, long-term morbidity for the infant, which could also lead to several chronic, degenerative diseases later in life (van den Broek *et al.*, 2005; Beck *et al.*, 2009; Svensson *et al.*, 2009; Goldenberg & McClure, 2010).

There are several factors that can have an impact on pregnancy outcome. Maternal demographics, such as race, education, socioeconomic status, marital status and frequency of vaginal or intra-uterine infections, are probably the most important factors that could pose a health risk to mother and infant (Filho *et al.*, 2010; Marrazzo, 2011). It is important to identify factors that could predispose women to BV acquisition to enable screening of those at risk, and therefore prompt administration of necessary treatment (Thorsen et al., 2006). A possible explanation for race being a risk factor for BV acquisition can be attributed to the differences in functioning of the hypothalamic-pituitary-adrenal (HPA) axis in black women compared to white women. The HPA axis is respomsible for controlling the human stress response, and is regulated by corticotrophin-releasing hormone (CRH). During pregnancy, CRH is produced in the placenta, decidua and foetal membranes. In addition to having a higher concentration of CRH, there are several CRH polymorphisms present in black women only, that contributes to their increased risk for BV (Mastorakos & Ilias, 2000; Ryckman *et al.*, 2009). In our study, the locations from

which participants were recruited, was an indication of the population groups they served, the majority of which live under challenging socio-economic conditions. Although we did not have refined data on the ethnicity of participants, we do know that the four locations serve mainly black and coloured communities. Taking this into consideration, we were able to demonstrate an association between a history of PTD and location of population. It has previously been demonstrated that black women have approximately double the chance of delivering preterm compared to their white counterparts (Goldenberg & McClure, 2010; MacDorman, 2011). In addition to a general lack of resources in black communities, the possible role of specific gene polymorphisms associated with African ancestry, have been the attributing factors (Anachebe, 2006; Tsai *et al.*, 2012).

Compounding the problem is that low socioeconomic status is significantly associated with an increased prevalence of BV among African American women (Paul *et al.*, 2008). Socioeconomic status plays a huge role in whether or not a woman will have a successful pregnancy. If she is uneducated, she will not likely be able to make informed decisions regarding prenatal care. Likewise, if she is unemployed or holds a low income job, she will not have the necessary access to adequate prenatal care. Although our study population was reasonably well educated, they generally held low income jobs. Access to free antenatal care, reduced the risk for PTD in this study population, although other socioeconomic factors may have been implicated. Previous studies in Southern Africa detected BV in 52% of pregnant women from a mainly black underprivileged population in Kwa-zulu Natal, (Govender *et al.*, 1996), whereas in The Gambia, a BV prevalence of 47.6% was reported (Demba *et al.*, 2005). Govender *et al.* (1996) attributed their high incidence to low socio-economic conditions often linked to promiscuity. Although there are definitely demographic similarities between Govender's study population and the current study population, the two studies were performed in different regions of the country. In addition, Govender's study was performed more than ten years ago and since then, there has been a great improvement in the health system in this country, as well as a greater body of knowledge with regard to women and pregnancy issues. According to data obtained from National Health and Nutrition Examination Survey (Allsworth and Peipert, 2007), BV was 3.13 times more prevalent in African American women than non-Hispanic white women. However BV was just as prevalent in our "black" population as the general American population.

Sexual behaviour, more specifically number of sexual partners and age of first intercourse, have been associated with an increased prevalence of BV. In our study, provision was not made to obtain such information as it was not required to fulfil the objectives. We did however collect information pertaining to relationship status. In the study, "single" was regarded as women not currently in a relationship. More women (48.2%) fell into this category than those who were married (44.5%). We observed BV more frequently in single women, who could possibly have had multiple sexual partners, thereby supporting the notion that BV is associated with increased number of sexual partners (Allsworth & Peipert, 2007). However, in the past, being married was an indication that a couple is monogamous and therefore not at risk of sexually transmitted infections (STIs) associated with promiscuity, but this is no longer necessarily the case. Even though infections may not always produce symptoms, abnormal vaginal presentation is generally a concern during pregnancy as it could be seen as a risk factor for PTD.

The general prevalence of HIV-positive subjects was low (24.58%), with a few not willing to reveal their HIV-status. In addition, most participants (72.43%) claimed not to have had an STI previously; therefore HIV status and previous STI could not be associated with a history of PTD. However clinical symptoms, such as vaginal discharge, were strongly associated with a history of PTD, as more than half of participants presented with a vaginal discharge. Although the frequency of BV was low in HIV positive subjects (13.9%), we were able to demonstrate an association between BV and HIV status. However; BV did not appear to affect a woman's chance of acquiring HIV in this population.

Previous studies have indicated an increase in the prevalence of BV with age (Larsson *et al.*, 2007) . In our study, as with the study by Allsworth & Peipert (2007), we found BV prevalence to decrease with age. The difference between this study and those indicating increased BV prevalence with age, is that while we sampled only pregnant women, their subjects included all women attending sexually transmitted disease clinics. STIs are generally more common among younger women, therefore the fact that BV prevalence increases with age has been used to defend the argument that BV is not a STI (Larsson *et al.*, 2007). We now know that although it may be sexually transmitted, its aetiology is due to an alteration in the balance of the vaginal micro-flora. Therefore BV may be considered a "sexually enhanced disease", with frequency of intercourse playing a vital role in BV transmission (Verstraelen *et al.*, 2010).

In this study, age was not associated with adverse outcomes, but it appears increased parity may be associated with PTD. It is recognized that the interaction between maternal age and parity is a risk factor for adverse perinatal outcomes, including preterm birth (PTB), such that young women who have given birth more than twice and older women experiencing their first pregnancy have greater risks (Schempf *et al.*, 2007).

Very few of our participants were smokers (13.62%) or alcohol consumers (9.63%). Although both prenatal alcohol consumption and maternal smoking have both been shown to increase the risk of PTD, (Ng *et al.*, 2006; Aliyu *et al.*, 2010), we were only able to demonstrate an association between smoking and a history of adverse pregnancy outcomes in this study. In addition, smoking also increases the risk of BV acquisition through various possible pathways. Smoking not only curbs the growth of lactobacilli (Livengood, 2009), but it also affects the genetic vulnerability for BV development as a result of its ability to affect stress-related genes (Ryckman *et al.*, 2009). However, as a result of a small number of reported smokers in our study, smoking was not indicated as a risk factor for BV for this population.

Observational studies have demonstrated a strong association between vaginal douching and bacterial vaginosis. Douching is a process that involves rinsing the vagina as a means of cleansing it. This process is risky as it changes the delicate chemical balance and micro-flora composition in the vagina, in so doing, making a woman participating in this practice more prone to BV. It is not clear whether douching increases the risk of BV or whether BV symptoms lead women to douche (Brotman *et al.*, 2008; Luong *et al.*, 2010). A great number of our study population did not respond to the question pertaining to douching, perhaps because they were not familiar with the practice. As a result we could not establish an association between douching and BV, as data was insufficient. We were however able to get an indication of the level of personal hygiene practiced by participants. Many of them reside in informal settlements where proper sanitation is lacking. Even though we observed an increased prevalence of BV in women participating in hand washing in a wash basin compared to bathing or showering, this was not associated with an increased chance of developing BV.

4.2.   PREVALENCE OF *G. VAGINALIS* IN BV

Upon sample collection, participants were examined for clinical signs of infection, including urinary tract infections and vaginal discharge**,** both of which are a major risk for PTD (Onderdonk *et al.*, 2003; Menon *et al.*, 2011).

The literature is filled with reports on the relationship between infections of the lower genital tract and spontaneous preterm birth. While numerous available reports are conflicting, BV has consistently been associated with adverse pregnancy outcomes, including preterm labour, pelvic inflammatory disease, and delivery of low birth weight infants. BV is a surprisingly prevalent condition, affecting approximately 3 million women annually (Wang, 2000). The exact sequence of events that causes the onset of BV is unknown, but BV may possibly have a multi-factorial aetiology and occurs as a result of microbial colonisation and alteration that leads to inflammation of the upper genital tract (Kimberlin and Andrews, 1998).

Generally Amsel's criteria are implemented to diagnose BV within a clinical setting. A patient is considered positive for BV if any three of the four Amsel criteria are met. However, the aim of this study was to determine the accuracy of Nugent scoring and culture to predict BV, and as a result of time and resource limitations, we were unable to execute all of the Amsel criteria in making a clinical diagnosis of BV and instead examined for "clue cells" on Gram-stained slides in conjunction with Nugent scoring. A subject was positively diagnosed with "clue cells" if ≥20% of epithelial cells were studded with variable Gram-positive coccobacilli.

We followed the suggestion by Spiegel (1991) to use Kopeloff's modification of the Gram stain with a safranin counterstain, as it aids in differentiating *G. vaginalis* from *Bacteroides* spp. The Kopeloff stain minimizes overdecolorisation of Gram-positive bacteria while simultaneously increasing the visibility of Gram-negative organisms (Libman *et al.*, 2006). Microscopic examination of Gram-stained slides is considered an accurate means of diagnosing BV (Schmidt and Hansen, 2000). Gram stain score and diagnosis by the clinical criteria has low agreement. While Gram stain quantification is more sensitive, Amsel criteria are more specific (Livengood, 2009).Therefore Nugent scoring was selected to diagnose BV in this study. Clue cells were observed in 25.2% of slides examined, while BV was diagnosed from vaginal swabs in 31.13% of pregnant women with a history of preterm delivery and 23.7% of women

with a history of FTD, thereby indicating that not all BV-positive patients will demonstrate clue cells. Although less than half of subjects diagnosed with BV by Nugent scoring presented with "clue cells", the two factors were highly associated with each other. Contrary to previous reports, in this study "clue cells" presented as a more specific (96.7%) indicator for BV, with very low sensitivity (32.3%). In support of this, it is worth noting that when clinically examined, asymptomatic women may not present with a discharge, amine odour may be masked by cervical mucus or blood and, while increased vaginal pH may be a sensitive predictor of BV, it is also characteristic of other vaginal conditions (Goyal *et al.*, 2005; Kwasniewska *et al.*, 2006).

In this study, Nugent scoring showed good specificity and sensitivity in diagnosing BV compared to culture techniques. Even though this study did not examine for other bacterial species associated with BV, our results support reports suggesting the crucial role of *G. vaginalis* in BV development. In a comparative study (Krohn *et al.*, 1989), diagnosis by clinical criteria was compared to Gram stain, gas-liquid chromatography, and *G. vaginalis* culture. Of the women diagnosed with BV by finding three of the four Amsel clinical signs (21%), only 12% were diagnosed by Gram stain, 28% by gas-liquid chromatography, and 41% by *G. vaginalis* culture. Since *G. vaginalis* is highly prevalent in women with BV, culture for *G. vaginalis* has a high sensitivity for predicting a diagnosis of BV while Gram staining vaginal smears is more specific because a positive culture may also be obtained from healthy women.

Aerobic vaginitis (AV) refers to a vaginal condition where abnormal vaginal flora distinctly different from BV are observed. AV is characterised by the presence of aerobic micro-organisms such as group-B streptococci and *Escherichia coli*, and the absence of *G. vaginalis* (Donders, 2007). With the aid of a composite scoring system (Donders et al., 2002), AV was diagnosed in 59.6% of participants classified as "intermediate" using Nugent scoring. In accordance to with previous reports, AV was significantly associated with PTD in this study (Lietich & Kiss, 2007).

Although considered to be risk factors for PTD, microscopic examination for normal flora, intermediate flora, BV, AV and "clue cells" were not significantly different between PTDG and FTDG in this study.

Microscopy cannot reliably differentiate bacterial morphotypes; therefore culture-based techniques using selective media are often used as a supplement to microscopy (Donders *et al.*, 2009). Considering the polymicrobial nature of BV, the isolation of one micro-organism is not a specific indicator of BV

infection. In addition, culture techniques present with a number of shortcomings. It is time-consuming, requires the application of strict sample collection protocols, and is unable to isolate many of the species present in the biofilm observed in BV-positive patients (Srinivasan & Fredricks, 2008).

The PPV was calculated to examine the probability of a positive *G. vaginalis* culture indicating BV infection (as diagnosed by Nugent scoring) and if a positive Nugent score could predict a positive *G. vaginalis* culture. In this study, Nugent scoring more accurately predicted BV, compared to isolation of *G. vaginalis* by culture techniques. In addition, we observed an association of 84.68% between BV and *G. vaginalis*. In PTDG, the likelihood of *G. vaginalis* being isolated from mothers who were diagnosed with BV by Nugent scoring was 82.98%, whereas in FTDG it was 64.15%. This difference can be attributed to the fact that in FTDG, there were endogenous asymptomatic carriers of *G. vaginalis* and/or that their overall microbial consortia were of a less virulent nature.

4.3.   THE SIGNIFICANCE OF *G. VAGINALIS* IN BV

G. vaginalis has for a long time been implicated as the sole cause of BV playing a pivotal role in its development (Turovskiy *et al.*, 2011). However, it is also recovered from women with normal vaginal flora; thus casting doubt on its role in the pathogenesis of BV. Considering that *G. vaginalis* can be detected in healthy individuals, and that several other micro-organisms have been recovered from women diagnosed with BV, (e.g. *Atopobium vaginae*, *Prevotella bivia*, *Mobiluncus mulieris*, *Veillonella spp.*, *Peptostreptococcus spp.* and *Fusobacterium nucleatum)*, indicating *G. vaginalis* as the dominating pathogen in BV would not be entirely accurate (Tabrizi *et al.*, 2006; Biagi *et al.*; 2009; Patterson *et al.*, 2010; Diao *et al.*, 2011), although it appears to be essential in the development of the BV biofilm.

With that said several researchers have studied the relationship between BV and *G. vaginalis* and consistently isolated *G. vaginalis* from women diagnosed with BV (Holst *et al.*, 1994; Goyal *et al.*, 2005). Despite that fewer women diagnosed with BV were colonised with *G. vaginalis* in this study (40.6%), compared to the 87.5-100% reported by others (Aroutcheva *et al.*, 2001; Bradshaw *et al.*, 2006; Hale *et al.*, 2006), *G. vaginalis* remains to be strongly associated with BV. How and why *G. vaginalis* plays such a critical role in the development of BV eluded many researchers, but with the development of advanced molecular techniques, the answers are becoming clearer. Analysis of vaginal biopsies revealed that BV is characterized by a dense biofilm on the vaginal epithelium dominated by *G.*

vaginalis as well as other fastidious anaerobes. (Kimberlin and Andrews, 1998; Bradshaw *et al.*, 2006; Allsworth & Peipert, 2007; Biagi *et al.*, 2008; Menard *et al.*, 2008; Marrs *et al.*, 2012).

The dominant role of *G. vaginalis* is attributed to its vast arsenal of virulence properties. It boasts an ability to adhere to vaginal epithelial cells and form biofilms. It also demonstrates cytotoxicity and other factors essential to establishing an infection (Marrs *et al.*, 2012). Adherence is an essential function in the pathogenesis of BV, as it aids in avoiding host defence clearance. The ability of *G. vaginalis* to adhere to vaginal epithelial cells is also an important first step in the formation of a biofilm. As with a dental biofilm, where specific bacteria have an affinity for the tooth pellicle, thereby enabling other bacterial species to attach, similarly the adherence and bioflim formation by *G. vaginalis* allows avirulent opportunists, with minimal pathogenic potential, to become established in the vagina (Braga *et al.,* 2010; Patterson *et al.*, 2010). In addition, it has been shown that *G. vaginalis* has the ability to be internalised by vaginal epithelial cells. This intracellular localization, together with bioflim formation could allow *G. vaginalis* to escape the immune response, as well as exhibit antibiotic resistance (Marrs *et al.*, 2012). Furthermore *G. vaginalis* produces phospholipase C and protease (Udayalaxmi *et al.*, 2011) that are able to damage oviduct mucosal surfaces and disable cilia function that could possibly lead to infertility (Taylor-Robinson and Boustouller, 2011). *G. vaginalis* also produces haemolysin, which serves as a food source for other bacterial species, and vaginolysin, a cytolysin that activates the protein kinase pathway in human epithelial cells resulting in subsequent cell death (Rottini *et al.,* 1990; Gelber *et al.*, 2008; Patterson *et al.*, 2010). The role of vaginolysin in the pathogenesis of BV has been supported by the production of IgA antibodies against vaginolysin (Cauci *et al.,* 2003).

Even though virulence properties of *G. vaginalis* are not associated with a specific biotype, specific biotypes are associated with BV (Udayalaxmi *et al.*, 2011). In this study, Biotype 7 was more frequently detected in participants diagnosed with BV. However, Biotype 2 occurred more frequently in women with a previous PTD (19.2%), while Biotype 4 was detected significantly more frequently in those with a history of FTD (18.4%). A previous study did not discriminate between specific groups, but associated Biotype 5 with BV; even though there were discrepancies in what was said in-text and what was represented in results (Briselden & Hillier, 1990). Conversely, Aroutcheva *et al.* (2001) clearly illustrated Biotypes 7 and 8 to be associated with BV.

Although biotyping is an old method of characterising *G. vaginalis*, with the aid of more advanced technologies, the specific tests for hippurate hydrolysis, lipase and β-galactosidase activity has improved. Unfortunately, the amount of available data on prevalence of biotypes in different regions is minimal and outdated. Biotype 5 was detected in all regions previously studied, except Bosnia (Piot *et al.*, 1984; Briselden & Hillier, 1990; Numanovic *et al.*, 2008; Udayalaxmi *et al.*, 2011). Overall Biotype 7 was the predominant biotype in this study, followed closely by Biotype 5. In women with a history of PTD and diagnosed with BV and AV, however, Biotypes 2 and 5 were increased while Biotype 4 was markedly decreased. The only difference between Biotype 7 and Biotype 5 is the ability of Biotype 5 to hydrolyse hippurate.

4.4. METRONIDAZOLE & CLINDAMYCIN RESISTANCE OF *G. VAGINALIS*

Considering that BV increases the risk of PTD, there is a need for effective treatment strategies to reduce the PTD rate. Antibiotic therapy has shown success in eliminating BV in pregnancy (McDonald *et al.*, 2007). The Centres for Disease Control recommends either oral or intravenous administration of Metronidazole or Clindamycin. Administration of these regiments has shown good success, but only for a short term. Approximately 30% of BV cases show recurrence at 3 months and approximately 50-80% at 1 year following therapy with either antibiotic. Recurring infection has been attributed to *A. vaginae* showing erratic susceptibility patterns to Metronidazole, the ability of *A. vaginae* and *G. vaginalis* bioflim to survive antimicrobial attack, as well as re-infection by another biotype (Menard *et al.*, 2011).

The big problem with re-administration of a similar antimicrobial is the chance of developing resistance. Metronidazole acts against anaerobic bacteria only and has therefore been the preferred choice of treatment for BV as it does not disturb the natural vaginal ecosystem (Austin *et al.*, 2005; Livengood, 2009). Since the early 1980s, Metronidazole has been used successfully to treat BV, but over the last decade Metronidazole resistance has become a frequent occurrence, including against *G. vaginalis* (Austin *et al.*, 2005; Nagaraja, 2008; Tomusiak *et al.*, 2011). This phenomenon was also observed in this study with Metronidazole resistance seen among 99% of *G. vaginalis* isolates. *A. vaginae* has been identified as the culprit for the Metronidazole resistance observed in BV-positive patients (Shopova *et al.*, 2011) and although Metronidazole has been used successfully to treat BV, the last decade has revealed Metronidazole resistant strains *G. vaginalis* not previously detected.

In women with a history of PTD, Biotypes 2, 5 and 7 was significantly resistant to Metronidazole, while biotypes 3, 4 and 8 showed Metronidazole resistance in those with a previous FTD. These findings correspond well to that of Aroutcheva *et al.* (2001). The resistance of *G. vaginalis* to Metronidazole in this study clearly indicates that the resistance to Metronidazole in the treatment of BV may be attributed to resistant strains of *G. vaginalis* and not only *A. vaginae* as previously reported. Compounding the problem is the emergence of Clindamycin resistant *G. vaginalis*. Although Clindamycin resistance was much lower (24%) than Metronidazole, in this study, in addition to Biotype 2, our findings are in accordance to that of Nagaraga (2008), who demonstrated Biotypes 4 and 5 showing Clindamycin resistance.

As a result of resistance being on the rise, alternative therapy options have been investigated. Recently, Togni *et al.* (2011) demonstrated the in vitro effectiveness of Nifuratel which showed activity against the consortium of bacteria recognized in BV development, while simultaneously not harming the normal lactobacilli flora. Currently, two clinical trials are ongoing to compare it to standard BV treatments (Togni *et al.,* 2011). The use of probiotics may change the way prophylactic treatments are conducted in the future since they pose no threat to either mother or foetus.
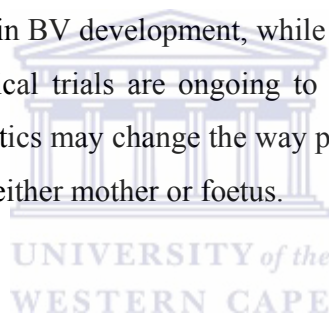
4.5.   SUMMARY & CONCLUSION

In this study, risk factors for BV were identified and the accuracy of microscopy and culture for the diagnosis of BV were compared. Nugent scoring and the presence of "clue cells" did not show a significant difference between PTD and FTD groups. However, a significant association was detected when BV was compared with the isolation of *G. vaginalis* biotypes in the two groups ($p = 0.000$). While Biotype 7 specifically is particularly associated with BV, Biotype 2 appears to be associated with BV in women with a previous PTD.   In addition, Metronidazole-resistance was strongly indicated in the *G. vaginalis* strains isolated, with Clindamycin resistant strains emerging and the perceived role of *G. vaginalis* in BV confirmed. The null hypothesis is therefore rejected as *G. vaginalis* was isolated significantly more frequently in women diagnosed with BV.

Attempts to recover many of the patients' folders *post partum* in order to establish their pregnancy outcomes were unsuccessful. For this reason, the results presented in this study are limited to patient history of previous PTD or FTD only and are therefore not associated with the pregnancy outcome of the studied gestational period.   Failure to obtain the follow-up data may be attributed to:

i. Obtaining permission from the correct authority to access patient folders. Each clinic has its own management who are not freely available for consultation, nor do they respond to written requests. Without their permission, we could not have access to many of the folders.

ii. Most clinics are under-staffed with the result that staff are pushed to the limit and are not able to assist beyond their call of duty, even though many would like to do so.

iii. Heavy workloads create inefficiencies regarding administration resulting in patient folders being misfiled, lost or not updated.

iv. Many patients do not deliver at the clinic where they received antenatal care**,** and thus, even if the folder was available, pregnancy outcomes were not recorded**.**

This limitation is indeed a pity since, had we managed to overcome these difficulties, it would have strengthened the predictive value of the study. An extension of this study to include the current pregnancy outcomes remains an option for future research.

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Appendix A: Questionnaire

Sample number: _____

Folder number: _____ KMOU ☐   MPP☐   GUG ☐

Residence: _____

Age: _____   **Grav**: _____   **Para**: _____

Height:_____ **Weight**: _____

PAST OBSTETRIC HISTORY

Year	Gestation/Birth weight[1]	Complications[2]	Neonatal Outcome[3]

Please code as follows:

1. **GESTATION/BIRTHWEIGHT**

500-999g	1
1000-1499g	2
1500-1999g	3
2000-2499g	4
2500g+	5
unknown	6

2. **COMPLICATIONS**

Extreme multi-organ immaturity	1
Hyaline membrane disease	2
Intraventricular haemorrhage	3
Necrotizing enterocolitis	4
Pulmonary haemorrhage	5
Hypoxic ischaemic encephalopathy	6
Meconium aspiration	7
Septicaemia	8

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Pneumonia	9
Unknown	10

3. NEONATAL OUTCOME

Neonatal death	1
Admitted to NICU	2
Discharged	3

PAST MEDICAL HISTORY

Hypertension	1
Diabetes	2
Previous urinary tract infections	3
Other	4

If other, please specify:_____
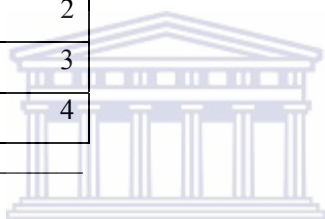
PAST SURGICAL HISTORY

Myomectomy	1
Ectopic	2
Other uterine surgery	3
Other non-gyneacological surgery	4

If 3, please specify:_____

If 4, please specify:_____

DEMOGRAPHIC & LIFESTYLE FACTORS

MARITAL STATUS

Single ☐   Married ☐   Separated ☐   Divorced ☐

Other (Please specify) _____

HIGHEST LEVEL OF EDUCATION Please tick (√) the correct box

G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12	UNIVERSITY	NONE

EMPLOYMENT　　　　　☐YES　　　☐NO

If yes, please specify: _____

SMOKING HABITS

DO YOU SMOKE?　　　　　　　Y ☐　　　N ☐

HOW MANY A DAY?　　<10 ☐　　10-20 ☐ >20 ☐

HOW LONG HAVE YOU SMOKED? _____

DO YOU SMOKE DURING PREGNANCY?　Y ☐　　　N ☐

DO YOU USE TIK?　　　　　　Y ☐　　　N ☐

DO YOU USE ANY OTHER SUBSTANCES?　　Y ☐　　N ☐

IF YES, PLEASE SPECIFY_____

ALCOHOL USE

DO YOU DRINK ALCOHOL　　Y ☐　　　N ☐

IF YES, WHAT DO YOU CONSUME?_____

HOW MANY DRINKS PER WEEK DO YOU DRINK?

　　　　　☐ <5　　☐ 5-10　　☐ >10

DO YOU DRINK DURING PREGNANCY?　Y ☐　　N ☐

REPRODUCTIVE HEALTH

HIV STATUS　POS ☐　　　NEG ☐　　　UNKNOWN ☐ CD4_____ HAART_____

HAVE YOU VER BEEN TREATED FOR A SEXUALLY TRANSMITTED DISEASE?　Y ☐　N ☐

HOW MANY SEXUAL PARTNERS DO YOU HAVE CURRENTLY? _____

IS THIS A NEW PARTNER?　　　　Y ☐　　　N ☐

CURRENT SYMPTOMS:　　　　☐ VAGINAL DISCHARGE　　　☐ URINARY SYMPTOMS

PERSONAL HYGIENE

BATH ☐　　　　SHOWER ☐　　　　HANDWASH ☐

OTHER _____

DOUCHING Y ☐   N ☐
OTHER VAGINAL PRODUCTS? _____

UNDERWEAR? ☐ NONE   ☐COTTON ☐NYLON
OTHER? _____

PERINATAL HEALTH SCREENING

1. Have you had some very difficult things happen in the last year?	**Yes**	**No**
2. Are you pleased about your pregnancy?	**Yes**	**No**
	Yes	**No**
3. Is your partner supportive?	**Yes**	**No**
4. Have you had problems with depression, anxiety or panic attacks before?	**Yes**	**No**
5. Is your partner or someone at home sometimes violent towards you?		

For total, add all shaded areas that are marked   **Total…………**