The prevalence of bacterial vaginosis in KwaZulu-Natal and its association with the vaginal immune response and shedding of HIV and HSV-2

Presented by

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DECLARATION BY SUPERVISOR

As the candidate's supervisor I, Prof. Adriaan Willem Sturm, agree to the submission of this thesis.

Signed: _____

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DECLARATION

I, Kavitha Naidoo, declare that,

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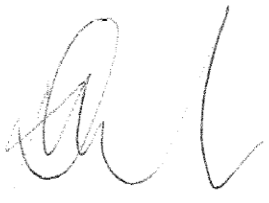
Signed: 

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DEDICATION

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ACRONYMS

BV -	Bacterial Vaginosis
EPS -	Exopolysaccharides
FRT -	Female reproductive tract
FISH -	Fluorescent In Situ Hybridisation
STI -	Sexually transmitted infections
PHC -	Primary healthcare clinic
SM -	Syndromic management
STD -	Sexually transmitted diseases
TPHA -	Treponema Pallidum Haemagglutination assay
RPR -	Rapid plasma reagin
LH -	Langerhans
DC -	Dentritic cell
Iqr -	interquartile ranges
VDM -	vaginal defined medium
IC -	Internal Control
LPS -	lipopolysaccharide

ABSTRACT

Introduction: South Africa has a high burden of sexually transmitted infections (STIs) and HIV. The role of *Gardnerella vaginalis* in the development of BV has been disputed after the recovery of *G. vaginalis* from healthy patients and the discovery of new bacteria using molecular identification. Infection with HSV has been associated with increased vaginal HIV RNA copies and bacterial vaginosis has been implicated as a risk factor for the transmission of HIV.

Methodology: Consenting patients of > 18 years were recruited from two different primary health clinics. Microscopy was used to diagnose BV and serology for HIV, HSV-2 and syphilis testing. *Chlamydia trachomatis* and *Neisseria gonorrhoeae* were detected by BD Probetec, and conventional PCR was used for the diagnosis of *Trichomonas vaginalis* and recognised ulcer pathogens. Quantitative bacteriology and HIV viral loads were done using the Applied biosystems ABI 7500 Real Time instrument. Immune cells from vaginal tampon specimens were analysed using flow cytometry.

Results: In both clinics, of the discharge pathogens *T. vaginalis* had the highest prevalence. The prevalence of both *T. vaginalis* and *N. gonorrhoeae* was significantly higher in Boomstreet clinic ($p<0.05$). The Umlazi D clinic had significantly more patients with BV ($p<0.0001$) and HSV-2 ($p<0.05$). Of the patients with ulcers, HSV-2 was detected in a one third of the specimens in each of the clinics. One patient was diagnosed with lymphogranuloma venereum (LGV). The Nugent score group 0-3 was dominated by *Lactobacillus* spp. while the Nugent score group 7-10 was dominated by *Gardnerella vaginalis.* The group with Nugent score 7-10 was shown to have significantly higher levels of immune cells that are proposed HIV targets. *Lactobacillus* spp. was associated with the group that was HIV antibody negative and *Prevotella* spp. with the HIV antibody positive group ($p < 0.05$). *Prevotella* spp. was not associated with shedding of HIV. The number of bacterial copies of *G. vaginalis* was significantly higher in patients shedding HIV ($p < 0.05$). In those shedding HSV-2 the number of copies of *G. vaginalis* was also higher but this did not reach statistical significance.

Conclusion: The trend in STI prevalence was similar to that described previously. We report circulating LGV and there is a possible increase in gonorrhoeae which needs to be confirmed. The potential pathogenic role of *G. vaginalis* in BV as well as the increased risk of HIV transmission is emphasized.

CHAPTER ONE

1.1 THESIS OVERVIEW

1.1.1 Study design and Methodology

Ethical approval was obtained from the Biomedical Ethics Committee of the University of KwaZulu-Natal (ethics number: BE475/14). Informed consent was given by all participants for the collection of specimens for research and diagnostic testing. Patients over the age of 18 presenting with vaginal discharge were recruited from the Boomstreet and Umlazi D Clinic which are primary health care (PHC) clinics situated in KwaZulu – Natal. These sites were chosen for convenience as previous studies by this same research group utilized these study sites and both staff and patients were accustomed to this study type. Pregnant women were excluded to avoid that patients would make a perceived link between participating in the study and adverse pregnancy outcome.

PHCs service the general population and employs syndromic management. As it is not a facility which treats patients for specific conditions, namely patients at high risk for STIs and BV, the data from this cohort is thus representative of the general South African population.

An illustration of the methodology is shown in Figure 1. A detailed description of the diagnostic tests and methodology employed are presented in the relevant chapters.

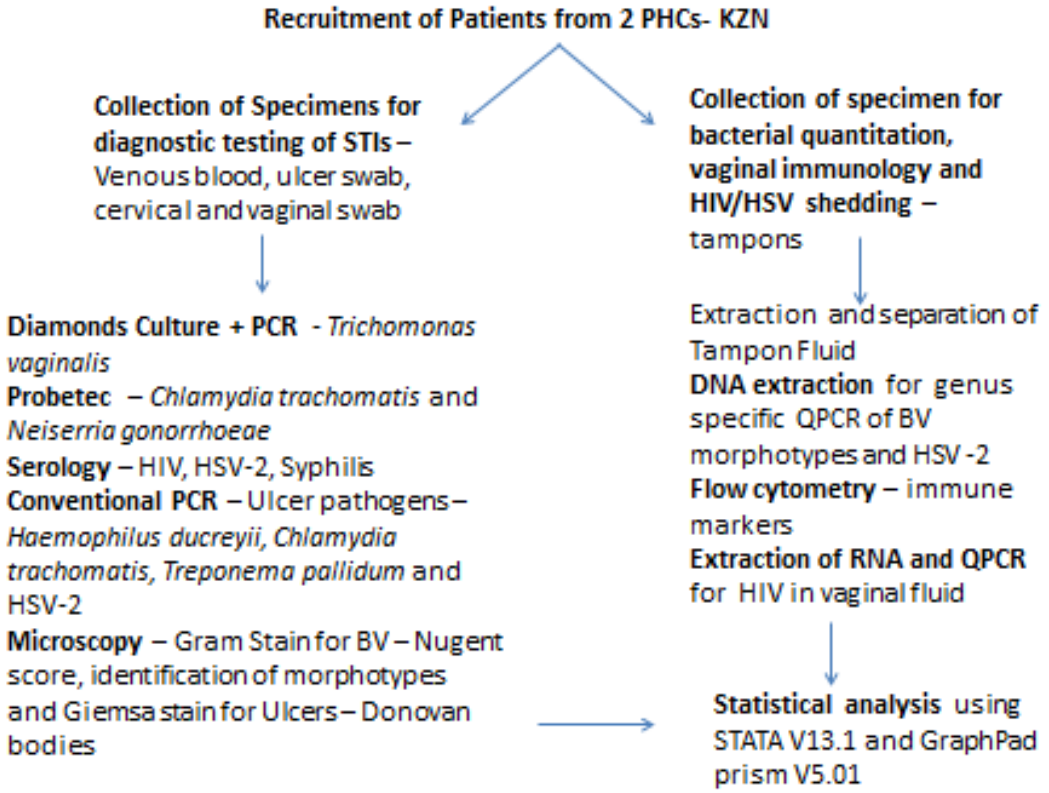

Figure 1: Diagrammatic presentation of methodology

1.1.2 Structure of PhD Thesis

This PhD thesis is structured according to the guidelines stipulated by the College of Health Sciences at the University of KwaZulu-Natal for the thesis by manuscript format. Each of the 3 manuscripts' are formatted according to the journal to which they are submitted. The thesis is guided by the following chapters:

Chapter 1: This chapter is a review of the literature and a presentation of the rationale of the study. The association of bacterial vaginosis associated bacteria in the transmission of HIV is described. Reported in vitro and in vivo observations define the vaginal immune response to associated bacteria, the effect of bacterial metabolic products on the vaginal microbiome and the relative risk of HIV transmission.

Chapter 2: This chapter is entitled: "**Prevalence of sexually transmitted diseases in women in KwaZulu-Natal"** which has been submitted to The Journal of Sexually Transmitted Infections and is currently under review (sextrans-2017-053338). The article details the geographical location of 2 primary healthcare clinics in Kwa-Zulu Natal from which patients were recruited. It describes the prevalence of sexually transmitted infections in each of these clinics and underscores the differences between the two. Additionally, the trend in STIs among women presenting with vaginal discharge in KZN over the last 26 years is highlighted and placed in the context of what is known for South Africa as a whole.

Chapter 3: In the previous chapter the STI burden of *N. gonorrhoeae* and *T. vaginalis* was significantly higher in the Boomstreet clinic while bacterial vaginosis and HSV-2 was higher in Umlazi D clinic. Infection with HIV remained constant. Literature supports the idea that bacterial vaginosis increases the risk of HIV transmission. The manuscript that forms this chapter focuses on a subset of women from the Umlazi clinic without a detectable STI. The bacterial morphotypes associated with bacterial vaginosis as identified by microscopy are described as well as the immune response related to both bacterial morphotypes and BV score in relation to HIV transmission. The manuscript in this chapter is entitled: **"*Gardnerella vaginalis* and immune response in patients with symptomatic bacterial vaginosis"** has been submitted to the Journal of Medical Microbiology and is currently under review (JMM-D-17-00465)

Chapter 4: Chapter 2 identified a group of symptomatic women attending a PHC in Umlazi in whom incident bacterial vaginosis and HSV-2 were significantly higher than in women attending a PHC in Boomstreet. The literature reports that HSV-2 increases the shedding of HIV. In chapter 3 the group with Nugent score indicative of BV had the highest relative percentage of *G. vaginalis*. It was found that women with vaginal flora dominated by *G. vaginalis* elicit an immune response with cell types that favor HIV transmission. The manuscript that embodies chapter 4 correlates the most frequently observed bacteria and the different Nugent score groups to the shedding of HSV-2 and HIV. The manuscript that makes up this chapter is entitled: **"Increased shedding of HIV and HSV-2 in patients with bacterial vaginosis is associated with high bacterial load of *Gardnerella vaginalis*"** has been submitted to the Journal of Sexually Transmitted Diseases and is currently under review.

Chapter 5: In this final chapter the relevant finding of the project in context with literature are discussed. The limitations are discussed and the conclusions emanating from this study are also presented.

1.2 INTRODUCTION

Bacterial vaginosis (BV) is characterised by a decrease in the amount of *Lactobacillus* species and an overgrowth of otherwise commensal bacteria. It is not considered a sexually transmitted infection although it shares many characteristics with this group of infections. It is a frequently occurring condition. The standard treatment is metronidazole. Recent molecular techniques have led to the identification of non-culturable bacteria such as *Atopobium vaginae* found to be resistant to metronidazole. *Atopobium vaginae* had a significantly higher prevalence in patients with BV as opposed to patients without, (Bradshaw et al. 2006), its resistance to metronidazole (Ferris & Masztal 2004) makes it an unlikely candidate as the cause of BV.

BV has been found to be associated with an increased risk of HIV1 transmission from female to male partners, (Cohen et al. 2012). Many studies suggest that BV associated organisms or their fermentation products increase propagation of HIV, (Cohen et al. 2012; Graham et al. 2013) In an in vitro study the expression of HIV was upregulated by *Peptostreptococcus asaccharolyticus, Prevotella bivia, Streptococcus agalactie,* and *Streptococcus constellatus*. However, *Lactobacillus acidophilus* did not upregulate the expression of HIV1, (Petrova et al. 2013). There is a greater diversity of microbial flora in HIV positive patients presenting with BV as compared to HIV negative patients with the same condition. In a separate study researchers found *Propionibacteriaceae, Anaerococcus,* and *Citrobacter* only in the HIV positive group with BV. These were absent in the HIV negative group and the HIV positive group without BV, (Spear et al. 2008).

The vaginal flora in healthy women has an abundance of *Lactobacillus* spp. that produce L-lactic acid which inhibits many other microbial species, (O'Hanlon et al. 2011). In addition, other studies have shown that cervico-vaginal mucus from women with a lactobacillus dominant vaginal microbiome was able to trap HIV allowing it to move 1000 fold slower than in water, (Lai et al. 2009). There is a proposed mutualistic relationship between *Prevotella* spp, a BV associated bacterial species producing butyric and succinic acid, and *Gardnerella vaginalis* as demonstrated by ammonia flow from *Prevotella* to *Gardnerella,* (Pybus & Onderdonk 1997). Butyric acid producing organisms have been shown to support the replication of HIV and its progression to AIDS, (Imai et al. 2009). Butyric acid has also been proposed to induce reactivation of latent HIV1 provirus, (Kenichi & Kuniyasu 2013). Therefore, the condition of bacterial vaginosis potentially increases the risk of transmission of HIV and the subsequent progression to AIDS.

With reference to pH changes in the vaginal environment brought on by BV associated bacteria, the range varies from an acidic pH of 4.5 to higher pH levels relative to colonisation by the different bacterial populations. Ongradi et al, (1990), found that infection of monocyte derived dendritic cells with HIV was inhibited at pH 5.7 when incubated for 2 hours. However, viral inactivation was achieved in 20 minutes when incubated at pH 5.4. Using a mouse model Olmsted et al, (2005) demonstrated a loss of motility of macrophages, monocytes and lymphocytes at a pH below 6, while T-lymphocyte activation was decreased at a pH of 4.5, thereby limiting the susceptibility of lymphocytes to HIV infection . Therefore it is important to determine the different bacterial species contributing to bacterial vaginosis and their effect on the pH of the vaginal microenvironment.

BV associated bacteria have been shown to affect immune responses, one effect being prevention of phagocytosis, (Mirmonsef et al. 2011). There is an abundance of macrophages present in mucosal sites. These macrophages are believed to harbour HIV and maintain a reservoir of infection, (Sharova et al. 2005). Bacterial vaginosis associated bacteria are known to produce short chain fatty acids such as butyric acid, acetic acid, etc. While the effect in the genital tract of these short chain fatty acids is unclear, investigation in the gut have revealed that these short chain fatty acids increased the production of pro-inflammatory cytokines by monocytes, (Mirmonsef et al. 2011).

In the study by Cassol et al, (2013) M2a monocyte derived macrophages were shown to express high levels of DC-SIGN which resulted in the increased ability of M2a to bind to HIV and become infected. This resulted in the transmission of virus to CD4+ T-cells despite low expression. The polarization of macrophages to M2a stimulation and the subsequent upregulation of DC-SIGN favor a TH2 response resulting in increased infectivity and replication of HIV 1.

In vitro experiments by St. John et al, (2007) found that vaginal fluids from patients with bacterial vaginosis activated and induced maturation of monocyte derived dendritic cells. They further demonstrated an increased production of IL12 when monocyte derived dendritic cells were exposed to cervical lavage fluid of patients with bacterial vaginosis. It is well documented that HIV binds receptors on dendritic cells, (Geijtenbeek et al. 2000). Stimulation of dendritic cells by BV associated bacteria drives maturation of these cells which then travel to the lymph nodes to transmit the virus to CD4+ T cells.

Microscopy remains the gold standard in laboratory diagnosis of BV. While the standardised scoring system by Nugent et al, 1991 is widely used, it is based on the assumption that Lactobacillus is dominant in healthy women while the increased presence of *Gardnerella vaginalis* is weighted heavily in patients with BV. Studies have reported asymptomatic women with a vaginal environment not dominated by lactobacillus, (Anahtar et al. 2015; Ravel et al. 2011). Advancement in microscopic techniques such as Fluorescent In Situ Hybridisation (FISH) have allowed for morphological characterisation of non culturable organisms identified by molecular techniques, (Fredricks et al. 2005).

Due to the growing evidence that bacterial vaginosis may be associated with an increased risk of HIV transmission and disease progression, in this study we aimed to morphologically identify bacterial types in a cohort of patients presenting with vaginal discharge regardless of HIV status. Quantifying the identified bacterial types will provide information regarding the dominant bacterial populations within a South African cohort of BV patients.

In addition, we looked at the viral load in the vaginal environment to determine whether a specific population of bacteria increased HIV shedding. By evaluating the presence of the different immune cells in vaginal fluid we plan to elucidate the immune response in patients representing the three states described by Nugent et al, (1991).

1.3 LITERATURE REVIEW

1.3.1 Pathogenesis of Bacterial Vaginosis

Bacterial vaginosis (BV) is defined in two different ways. The first one is mainly based on clinical signs and symptoms known as Amsel's criteria, (Amsel et al. 1983) The second one is based on observations regarding the composition of the vaginal microbiome, observed on a Gram stained smear. The most frequently used score system is known as Nugent's criteria, (Nugent et al. 1991) The microscopic definition identifies more women as having BV than the clinical definition. The vaginal microbiome is complex in its composition and its regulation to maintain a harmonized environment. Several studies have focused on the pathogenesis of BV and although a number of organisms have been implicated, no single organism has been identified as the causative agent. The mainstay in the development of BV is the shift in colonization with *Lactobacillus* spp. to other bacterial morphotypes, (Biagi et al. 2009). Some

women are symptomatic presenting with malodorous discharge, other women remain asymptomatic for reasons unknown, (Marrazzo 2011).

1.3.1.1 Factors associated with a 'healthy' vaginal microbiome

A normal, healthy vaginal microbiome is thought to comprise predominantly of *Lactobacillus* spp. In such women, the microbiome is dominated by lactic acid producing bacteria which acidify the vaginal environment to pH 3.5 to 4.5, (Aldunate et al. 2015). Asymptomatic women of different ethnic groups from North America were reported to be colonised by different *Lactobacillus* spp, (Ravel et al. 2011). This differential species dominance accounted for varying pH of the vaginal contents. Anahtar et al, (2015), in their study population of HIV negative asymptomatic Black women from South Africa found that only a minority had lactobacillus dominant vaginal microbiomes. Ravel et al, (2011), reported that 61.9% of their population of Black women in the US had lactobacillus dominance while 38.9% had a diverse microbial community. Lactobacillus dominance was found in 59.6% of Hispanic women. The authors concluded that in Black as well as Hispanic women the absence of a dominant lactobacillus community represents a normal vaginal state.

1.3.3 Shift in bacterial composition and the development of bacterial vaginosis

When there's a significant reduction in lactic acid producing *Lactobacillus* spp. and an increase in other bacteria, more especially anaerobic bacteria, the vaginal pH increases and there is development of BV. The increase in pH is attributed to the short chain fatty acids produced by anaerobic bacteria, (Aldunate et al. 2015).

Biagi et al, (2009), found a decrease in lactobacillus DNA in asymptomatic BV patients when compared to patients without BV and those with *Candida albicans* infection. Bacterial DNA of the BV associated anaerobes was increased in patients with asymptomatic BV. Their analysis showed synergistic relationships with increased DNA of different anaerobes in BV but could not elucidate a predominant bacterial type. They also found that the lactobacillus dominance remained during infection with *Candida albicans*.

Early studies attributed the shift to BV to the presence of *Gardnerella vaginalis,* (Gardner & Dukes 1955). The study of asymptomatic South African women by Anahtar et al, (2015) found only 37% had lactobacillus dominant communities and 45% of the remaining 63% had *G. vaginalis* dominance.

The frequent isolation of *G vaginalis* from healthy women, (Aroutcheva et al. 2001) throws doubt on the perception that this organism is the single organism responsible for the condition of bacterial vaginosis. However, in healthy patients the presence of *G. vaginalis* is present in smaller quantities as opposed to patients with BV indicating that bacterial load plays a role in BV, (Ratnam & Fitzgerald 1983). Different strains of *G. vaginalis* have been found to produce substances that act either as antagonists suppressing the growth of other bacteria or allow a synergistic relationship increasing the bacterial diversity associated with BV, (Teixeira et al. 2010).

In the study by Anahtar et al, (2015) the genus *Prevotella* was found to be present in microbial communities in the cohort of women with the greatest bacterial diversity .
Prevotella intermedia is associated with periodontal disease, (Yamanaka et al. 2009) and much like BV the anaerobic environment in the mouth allows for propagation of a diverse group of anaerobes. Their metabolic products influence the pH of the environment. Guan et al, (2006) found that *P. intermedia* was able to optimally degrade hemoglobin at pH 5.0, however degradation activity was observed over a wide pH range. Degradation of hemoglobin is essential for its survival as the organism is unable to produce its own source of heme. In addition *P. intermedia* was found to produce succinate, acetate and formate. The authors associated the production of the fatty acids with a drop in pH observed during bacterial growth. They concluded that the virulence of *P. intermedia* is based on the production of metabolic products, which are capable of decreasing the pH to levels which are favorable to the proteolytic enzyme required for degradation of hemoglobin. A similar mechanism can play a possible role in the development of BV.
In vitro experiments suggested a symbiotic relation between *Prevotella bivia* and *G. vaginalis.* Ammonia rich supernatants from *P. bivia* cultures were able to stimulate the growth *of G. vaginalis,* (Pybus & Onderdonk 1997).

1.3.2 The vaginal immune response

The female genital tract is under constant microbial challenge due to changes in diversity brought on by hormones, age, sexual activity etc. Anahtar et al, (2015), found that BV in general is not an accurate predicator of a specific inflammatory response. Instead, specific bacteria are capable of eliciting an immune response. In vitro analysis using a vaginal epithelial cell line identified four key bacteria showing significant differences in inflammatory cytokine production when compared to *Lactobacillus crispatus*. *Prevotella amnii* was found to induce significant levels at very low colonies numbers, (Anahtar et al. 2015).
Mice were infected with either exopolysaccharides (EPS) producing *P. intermedia* or non-EPS producers. The authors found that the EPS producing *P. intermedia* were phagocytosed less frequently than non-EPS producers indicating an ability to evade the immune response, (Yamanaka et al. 2011). In the vaginal tract, *Prevotella* spp. is present consistently. This indicates that the BV associated *Prevotella* spp. is able to evade the immune response, conserving their presence in the vaginal tract.

The pro inflammatory cytokines Il6 and IL8 as well as IL1 RA and the inflammatory cytokine MIF had higher expression in the female reproductive tract (FRT) cell lines (VK2, Ect1 and END1) when subjected to infection with *Atopobium vaginae* and *G. vaginalis* as compared to *L. johnsonii*. *A. vaginae* induced greater cytokine expression than *G. vaginalis* and the endocervix cell line END1 showed higher expression than the other epithelial cell lines, (Eade et al. 2012). This indicates that the FRT produces varying immune response relative to the type of epithelial cell present.

1.3.3 The association of BV in the transmission of HIV

The microbiological definition of BV is a shift in the vaginal microbiome resulting in a decrease of the amount of *Lactobacillus* spp. and an increase in anaerobic bacteria. *Lactobacillus* spp. are known to produce lactic acid capable of acidifying the vaginal environment to a pH that is not suitable for growth of other bacterial types, (Matu et al. 2010). Lactic acid rich cervical mucus has also been shown to trap HIV, (Lai et al. 2009). Asymptomatic ‘healthy’ women in South African have been shown to have a polymicrobial vaginal ecosystem, (Anahtar et al. 2015). The absence of lactobacillus dominance means a

decrease in lactic acid concentration and consequently in vaginal acidity. This in turn, results in greater infection capability and transmission of HIV, (Cone 2014).

A study of Rwandan sex workers showed that women having a lactobacillus dominant cervicovaginal micobiome were less likely to have STIs or HIV. Additionally, HIV positive women who had a high diversity cervicovaginal microbiome had higher detectable levels of HIV RNA, (Borgdorff et al. 2014). In the study by Anahtar et al, (2015), the group displaying high levels of pro-inflammatory cytokines also had significantly activated CCR5+CD4+ T cells. This group comprised 50% of BV positive women as diagnosed by Nugent's scoring and included the presence of the anaerobic bacterium belonging to the genus *Prevotella*. An in vitro experiment showing the adhesion capacity of different strains of *P. bivia* to a cervical epithelial cell line (HeLa) showed a varying degree of invasion between strains. The strains with the highest invasion capacity was associated with increased expression of IL6 and IL8, (Strömbeck et al. 2007). Interleukin 6 has been reported to upregulate the expression of HIV in monocyte derived macrophages (MDM), (Poli et al. 1990) while IL 8 was shown to increase the replication of HIV in vitro within MDM and peripheral blood lymphocytes, (Lane et al. 2001).

Some species of *Prevotella* including the periodontal pathogen *P. intermedia* were found to produce EPS, which represents a virulence mechanism. The authors reported that EPS producing strains of *P intermedia* were capable of producing abscesses in mice at much lower inoculum than non-EPS producers, (Yamanaka et al. 2011). Microscopic observation of biofilms on vaginal smears is a characteristic feature of BV. Biofilm formation is linked to the presence of bacteria capable of producing EPS, (Ghafoor et al. 2011). There is a likelihood that BV associated EPS producing *Prevotella* spp. could produce abscesses in the genital region.

The presence of bacteria associated with bacterial vaginosis in particular *G. vaginalis* and *Mycoplasma hominis* correlated with increased HIV RNA levels in CVL specimens, (Sha et al. 2017). A study of African couples found that the hazard ratio of men who acquired HIV infections was higher if their partner had BV compared to when the female partner had normal vaginal flora. BV was associated with an estimated three fold increased risk of HIV transmission, (Cohen et al. 2012).

1.3.4 Clinical and Laboratory characteristics of Bacterial Vaginosis

1.3.4.1 Clinical Diagnosis

Clinically the diagnosis of bacterial vaginosis is based on Amsel's criteria. This includes a set of 4 criteria of which 3 should suffice to diagnose BV. These criteria are the presence of a thin white homogenous vaginal discharge, vaginal pH greater than 4.7 but not higher than 5.5, a malodorous smell similar to that of rotten fish on the addition of 10% potassium hydroxide and the presence of 'clue cells' on wet prep microscopy which are small nucleated mature epithelial cells covered with bacteria, (Amsel et al. 1983).

1.3.4.2 Microscopic diagnosis of Bacterial Vaginosis

Bacterial Vaginosis (BV) has for a long time been defined as the decrease of the usually dominant *Lactobacillus* spp. and an increase of different bacterial morphotypes. Spiegel et al, (1983) found an inverse relationship between lactobacillus and gardnerella morphotypes during microscopic interpretation of vaginal smears. They proposed a grading system which defined healthy as those patients having 3 to 4+ lactobacillus regardless of the presence of gardnerella, while BV was the observation of reduced lactobacillus i.e lower than a score of 3 accompanied by mixed flora. This method had 100 percent agreement to clinical diagnosis of BV.

Later, Nugent et al, (1991) proposed a standardized scoring system. This system of grading followed that of Spiegel et al, (1983). This scoring was centered on the notion that *Lactobacillus* spp. was dominant in a 'normal' vaginal smear. Decreasing numbers of lactobacillus allows proliferation of 'other' morphotypes resulting in a shift from normal to intermediate finally resulting in bacterial vaginosis. This scoring system is still widely used today for the laboratory diagnosis of BV.

Begum et al, (2011) compared the use of acridine orange staining to Amsel's criteria and Nugent's scoring for the diagnosis of BV. This again was based on the presence of clue cells

which are epithelial cells covered with predominantly gardnerella. Gardnerella on epithelial cells stained orange yellow on the green epithelial cells. This stain correlated with Amsel's clinical criteria for the diagnosis of BV.

1.3.4.3 Molecular identification of bacterial types

Numerous studies have characterized the vaginal flora using molecular techniques, (Fredricks et al. 2005; Ling et al. 2010). In patients that lacked symptoms associated with BV the dominant bacterial type detected by 16S rDNA PCR was *Lactobacillus* spp. Bacterial sequences showing the progression to a bacterial vaginosis state in 2 asymptomatic patients who had a combination of Bacterial Vaginosis Associated Bacteria (BVAB) bacteria were detected by PCR at baseline. Using FISH, BVAB showed distinct morphological characteristics that separates them from other anaerobes and were also seen attached to epithelial cells in a way suggestive of clue cells, (Fredricks et al. 2005). The presence of *Atopobium vaginae* and *Gardnerella vaginalis* at a concentration of $\geq 10^8$ and 10^9 copies /ml were shown to be diagnostic of BV, (Menard et al. 2008) and high species diversity was associated with bacterial vaginosis. While a number of bacteria had a high relative abundance in patients with BV, bacteria designated BVAB 1, 2 and 3 were specific to bacterial vaginosis.
The relative abundance of *Lactobacillus* spp. is greater in healthy women than in patients with BV. Additionally there is enriched bacterial diversity in patients with BV, (Dols et al. 2016). The evaluation of bacterial diversity and decreased lactobacillus concentration could be used for diagnosis of BV. However; in resource poor countries this molecular approach would not be practical.

1.3.4.4 Presence of metabolites

Short chain fatty acids are reported in high concentrations in patients with BV. In an in vitro experiment by Al-Mushrif et al, (2000) succinic acid was shown to inhibit chemotaxis in monocytes derived from fresh human blood and MonoMac 6 cell line while lactic acid showed no inhibition. Gas chromatography analysis of bacterial culture supernatants showed that *Lactobacillus* spp. produced the highest level of lactic acid while *Prevotella* spp. and *Mobiluncus* spp. produced the greatest amount of succinic acid. Lactic acid was significantly decreased in patients with BV and succinic acid was significantly higher in patients with BV compared to healthy patients and those belonging to the intermediate group, (Al-Mushrif et al. 2000). In 1989, A.W Sturm reported the inhibition of white blood cell chemotaxis by 6

succinate producing anaerobes, with the greatest effect seen in the bacterial isolates producing the largest amount of succinate. The author postulated that non-specific vaginitis was likely caused by *Bacteroides ureolyticus* and *Mobiluncus* spp. due to its higher succinate production rather than *G. vaginalis*, (Sturm 1989).

1.4 AIMS, OBJECTIVES and HYPOTHESIS

Aim

To quantify bacterial genera representing the identified bacterial morphotypes in black South African women with vaginal discharge and to determine the effect of these microbes on HIV shedding and on the vaginal immune response.

Objectives

1. To determine the burden and aetiology of sexually transmitted infections (STI) in an urban population of Black South African women presenting with vaginal discharge
2. To determine by Gram stain microscopy the most frequently observed bacterial morphotypes in these women
3. To quantify using genus specific primers the bacteria representing the identified morphotype
4. To determine by means of flow cytometry the vaginal cellular immune response
5. To determine if there is an association between a specific bacterial genus, the presence of HIV infection and vaginal HIV and HSV-2 shedding

Hypothesis

Gardnerella vaginalis initiates a mucosal immune response in the cervico-vaginal system that favors the acquisition and transmission of HIV

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CHAPTER TWO

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"**Prevalence of sexually transmitted diseases in women in KwaZulu-Natal"**

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Prevalence of sexually transmitted diseases in women in KwaZulu-Natal

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ABSTRACT

Objective

South Africa is a developing country in which syndromic management is used to treat patients. The effectiveness of which is determined by the selection of antimicrobials based on local prevalence of the pathogen and the drug susceptibility profile. We report on aetiology of sexually transmitted infections in females presenting with discharge at two primary healthcare clinics (PHC) in KwaZulu-Natal.

Methods

Consenting patients > 18years were recruited. Microscopy was used to diagnose BV and serology for HIV, HSV and syphilis testing. *Chlamydia trachomatis* and *Neiserria gonorrhoeae* were detected by BD Probetec, and conventional PCR was used for the diagnosis of *Trichomonas vaginalis* and the recognised ulcer pathogens.

Results

In both clinics, of the discharge pathogens *T. vaginalis* had the highest prevalence. *T. vaginalis* and *N. gonorrhoeae* were significantly higher in the clinic situated at a taxi rank ($p<0.05$). The clinic in a residential area had significantly more patients with BV ($p<0.0001$) and HSV-2 ($p<0.05$). Of the patients with ulcers, HSV-2 was detected in one third of the specimens in each of the clinics. One patient was diagnosed with lymphogranuloma venereum (LGV).

Conclusion

There is a possible rise in gonorrhea. The prevalence of *N. gonorrhoeae* seen at the clinic at the taxi rank is higher when compared to previous reports on STI prevalence in South Africa. There is also evidence of circulating LGV.

Key words: STI, prevalence, syndromic management

Key messages:

- Prevalence of STIs and their aetiology differ between clinics depending on the location
- LGV is still present in the KwaZulu-Natal population

INTRODUCTION

The outcome of syndromic management (SM) of symptomatic sexually transmitted infections (STIs) depends on the accurate recognition of the syndrome,[1-2]. While the recognition of genital ulcer syndromes and male urethritis is highly accurate, this is not so for non-ulcerative STIs in women. Symptoms and signs of this syndrome include discharge from cervix and/or vagina, vulvo-vaginal itch , dysuria and lower abdominal pain,[3-4].
Women presenting with such symptoms to a primary health care clinic (PHC), are routed to the sexually transmitted diseases (STD) section of these facilities. The differential diagnosis for these symptoms includes sexually transmitted infections, non-sexually transmitted infections e.g. yeast vaginitis, malignancies, urinary tract infections or physiological discharge. Most patients, who present to a STD facility with genital ulcer disease and men with urethritis, are infected with a STI pathogen. In contrast, only a subset of women with non-ulcerative genital disease will have a STI.

Antimicrobial treatment for the different syndromes should be based on local data with respect to prevalence of the different pathogens and their susceptibility profile. Since these data change over time regular surveillance needs to take place. We report on the aetiology of female discharge syndrome in two different PHCs in KwaZulu-Natal.

MATERIALS AND METHODS

Patient recruitment and specimen collection

Consecutive consenting patients presenting with vaginal discharge were recruited on weekdays between 8:00 am and 12:00 from May till September 2014 at Boomstreet Clinic in Pietermaritzburg and from October till December 2014 at Umlazi D clinic in Durban in KwaZulu-Natal, South Africa. These clinics were chosen for convenience as both clinics had been used for previous studies and staff and patients were accustomed to this type of project.

Both are PHCs with an annual number of patients of 258 236 and 152 615 respectively. Ethics was approved by the Biomedical Research Ethics Committee of the University KwaZulu-Natal.

A vaginal smear was prepared by inserting a Dacron swab on a rigid shaft about 5 cm into the vagina, after which it was withdrawn while rotating. The swab containing the vaginal content was rolled onto a glass slide, thereafter placed into Diamonds media.

If an ulcer was observed, the slough was removed with dry sterile gauze and the resulting exudate was collected with a Dacron swab. The swab was placed into 1ml of PBS. A glass slide was placed onto the ulcer to prepare an impression smear.

A speculum was inserted into the vagina and the endocervical content was collected by inserting a female Probetec swab. A New York City (NYC) agar plate was inoculated on site before the swab was placed back into its holder and transported to the laboratory for DNA extraction.

Venous blood was collected from consenting patients for antibody tests.

Microscopic analysis

Vaginal smears were Gram stained and viewed by light microscopy using 1000x magnification for the diagnosis of BV. Slides were scored by 2 independent, trained readers using Nugent's criteria,(5). Discrepant scores were re-evaluated.

The impression smears were stained using a modified Giemsa stain and viewed using 1000x magnification for the presence of Donovan bodies.

The inoculated Diamonds medium was incubated at 37°C. After approximately 48 hours some medium was collected from the lower half of the broth column to produce a wet prep. This was examined under 400 x magnification for the presence of *Trichomonas vaginalis.* Negative cultures were re-incubated and the examination was repeated daily for another five days.

***Neisseria gonorhoeae* culture**

Inoculated New York City (NYC) plates were streaked to achieve single colonies and incubated for 48 hours at 37°C in air with 5 % CO_2 and saturated water vapour. Suspected colonies were tested for oxidase activity and oxidase positive colonies were subcultured. Identification to species level was done by Gram staining, catalase, oxidase and the production of acid from glucose.

Antibody detection tests

The Core 1 step HIV rapid test was used for the diagnosis of HIV1 and HIV2. The rapid plasma reagin test (RPR), (BD diagnostics, Sparks, USA) was used as a screening test for syphilis. Positive RPR tests were confirmed using the Treponema Pallidum Haemagglutination assay (TPHA), (Omega Diagnositics, Alva, United Kingdom). The Viricell HSV-2 IgG kit (Viricell, Granda, Spain) was used to detect antibodies against Herpes simplex virus type 2 (HSV-2). All these tests were performed as per manufacturer's instructions.

Molecular diagnostic testing

The endocervical swabs were processed using the BD Probetec ETAmplified DNA Assay (Becton Dickinson, New Jersey, USA) using Strand Displacement technology to detect *N. gonorrhoeae* and *C. trachomatis*,(6). *T. vaginalis* was detected by conventional PCR,[6-7] using the same DNA.

To release the ulcer material into the PBS, the swab was rotated against the side of the tube. DNA was isolated from 200 µl of the fluid using the QiaAmp DNA isolation mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA was eluted in 100 µl AE buffer. Extracted DNA was used as template for detection of *Treponema pallidum*, *Haemophilus ducreyii*, HSV-2,(7) and *C. trachomatis*,(8) by conventional PCR.

Statistics

Prevalence of infections is reported as the proportion of women with any one of the STIs or

BV. Differences between sites were assessed using the chi square test and, when applicable, Student's T-test. Data were analysed using GraphPad Prism version 5.01.

RESULTS

A total of 628 patients between the ages of 18 and 64 years were recruited from the two clinics: 373 (59%) and 255 (41%) patients at Boomstreet Clinic and Umlazi D clinic respectively. There were no differences in race, economic status or age between participants of the two clinic sites. At Boomstreet Clinic, 156 /373 (42%) were infected with one or more discharge pathogens, excluding HIV and BV compared to 75/255 (29%) at Umlazi D Clinic (p=0.002). In both clinics, *T. vaginalis* was the most frequently observed pathogen, followed closely by *N. gonorhoeae* and *C. trachomatis* (Table 1). The prevalence of *T. vaginalis* and *N. gonorrhoeae* was significantly higher at Boomstreet Clinic, while there was no difference between the two clinics for *C. trachomatis* (Table 1). There were significantly more patients with BV at Umlazi D as compared to Boomstreet (p< 0.0001). The prevalence of HSV-2 but not HIV was also significantly higher in Umlazi D (Table 1). At Boomstreet four cases of active syphilis were diagnosed with only one at Umlazi D.

Table 1: Prevalence of STIs in women presenting with vaginal discharge at two PHCs in KZN

	Boom street clinic		**Umlazi clinic**		
	n =	no. (%) positive	*n* =	no. (%) positive	*p* value
N. gonorrhoeae	372	66 (17.7)	253	27 (10.7)	0.01
C. trachomatis	372	53 (14.2)	253	28 (11.1)	0.25
T. vaginalis	373	79 (21.2)	255	32 (12.5)	0.005
BV > 7	373	164 (43.9)	255	153 (60)	< 0,0001
Syphilis serology ≥1:8; TPHA confirmed	99	4	70	1	0.82
Syphilis serology TPHA confirmed	99	9	70	2	0.80
HSV antibodies	99	66 (66.6)	68	55 (80)	0.04
HIV antibodies	99	60 (60.6)	70	45 (64.3)	0.98

n= the number of patients tested in each clinic

Thirteen women had concomitant genital ulcers, three at Boomstreet and 10 at Umlazi D. At both clinics, one third of these had a positive PCR for HSV-2: one out of three in Boomstreet and three out of ten in Umlazi D. One case of LGV was diagnosed at Umlazi D. Nine out of the 13 ulcers remained without an aetiological diagnosis.

DISCUSSION

The study presented here compares prevalence of the different causes of vaginal discharge syndrome of women attending two different clinics. Boomstreet clinic is situated at a taxi rank and will therefore be attended by patients from the area around the clinic as well as commuters from different parts of KwaZulu-Natal. Umlazi D clinic is situated in a semi-urban area with a stable population. The prevalence of *N. gonorrhoeae* and *T. vaginalis* was found to be significantly higher in Boomstreet clinic. Chlamydia infections have a more chronic nature as do HIV and HSV infection. The prevalence of the first two was similar in both clinics while HSV infections were seen with a slightly higher frequency at Umlazi D. For patients, there will be a direct link between the symptoms of infection with *N. gonorrhoeae* or *T. vaginalis* and sexual activity outside a stable relationship. Therefore, medical assistance would not easily be sought at the clinic in the area where people live. This may explain why these two infections are more prevalent in patients attending the clinic at the taxi rank.

Over the years many studies have been published addressing the prevalence of sexually transmitted infections in women. The results of eight studies conducted in KwaZulu-Natal over a period of 26 years are summarized in table 2.

Table 2: Aetiology of vaginal discharge syndrome in different cohorts in KwaZulu-Natal

	ANC clinic attendees Empangeni	**STD clinic attendees Hlabisa**	**ANC clinic attendees Hlabisa**	**Family planning clinic attendees Hlabisa**	**STD clinic attendees KwaMsane**	**ANC clinic attendees KwaMsane**	**HIV uninfected women Durban**	**Microbicide study participants Durban**
n=	193	140	271	189		245	242	2236
N. gonorrhoeae	6	6	8	4	12	7	5	3
C. trachomatis	11	6	13	7	15	11	4	11
T. vaginalis					36	32	20	10
BV					28	29	53	
Reference number	(9)	(10)	(11)	(12)	*	*	(13)	(14)

*Personal communication 2017, Prof A.W. Sturm – unpublished data
Prevalence indicated as percentage

Although the cohorts studied differ in the reason why they attended a healthcare clinic, the prevalence rates are similar. In the current study Infections with *N. gonorrhoeae* in Umlazi have the same prevalence as in the STD clinic in KwaMsane in 1999 but in Boomstreet clinic this is significantly higher. Whether this indicates that gonorrhea is on the rise needs to be established urgently. This is especially important if one takes into account the reports on cases

with 3rd generation cephalosporin resistant gonococci,[15-16] since ceftriaxone is currently used in the SM protocols. The prevalence of chlamydia infections has remained stable throughout the years while trichomoniasis seems to be decreasing. However, this might be a misrepresentation since the last two studies in table 2 have not been done in symptomatic patients. If we add the results of the study presented in this paper, then it seems that the prevalence of BV is increasing. Mhlongo et al, (2010) report prevalences of the same magnitude in patients with symptomatic STDs in Cape Town and Johannesburg with the exception of trichomoniasis which is with 36 % higher in Johannesburg than anywhere else.

Our study was not designed to elucidate the aetiology of genital ulcers. We only performed diagnostic tests when an ulcer was detected in a woman with vaginal discharge. We detected HSV2 in 4 of the 13 patients with ulcers while 9 had no detectable pathogen. This suggests that these were also genital herpes cases. The detection of one patient with chlamydia in the ulcer indicates that LGV is still present in our environment,(18).

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CONFLICT OF INTEREST

We declare that there is no conflict of interest.

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CHAPTER THREE

Manuscript entitled:

"*Gardnerella vaginalis* and immune response in patients with symptomatic bacterial vaginosis"

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Gardnerella vaginalis and immune response in patients with symptomatic bacterial vaginosis

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ABSTRACT

Purpose: The basis for laboratory diagnosis of bacterial vaginosis (BV) remains microscopy. We aimed to identify by microscopy the bacterial morphotypes most frequently observed and to quantify these genera by QPCR. Additionally we looked at the vaginal immune response at the time of collection and related this to the Nugent's score and bacterial quantities.

Methods: Based on Gram stain microscopy, participants were grouped according to Nugent's criteria. Genera representing the most frequently observed morphotypes were quantified by real time PCR (RT PCR). Immune cells from vaginal tampon specimens were analysed using flow cytometry. Statistical analysis was performed using STATA version 13.1.

Results: The Nugent score group 0-3 was dominated by *Lactobacillus* spp. while the Nugent score group 7-10 was dominated by *Gardnerella vaginalis*. The quantity of both organisms was significantly higher than all other genera in their respective score groups. The group with Nugent score 7-10 was shown to have significantly higher levels of immune cells that are proposed HIV targets.

Conclusion: *Gardnerella vaginalis* was found to dominate the group with Nugent score 7-10. This correlates with an immune response that favors HIV transmission. We found that in symptomatic patients the vaginal flora to be similar to asymptomatic women of African origin.

INTRODUCTION

Sexually transmitted diseases (STDs) increase the risk of HIV transmission. Although bacterial vaginosis (BV) is not considered a STD, it was shown that there was an association of BV with increased HIV transmission,[1,2]. HIV target cells include Langerhans (LH) cells, non-LH dendritic cells and macrophages. It is thought that HIV interacts with these cells in different ways under different conditions to promote viral spread,[3-4]. Determining the differences and changes from a healthy vaginal state to a BV state is paramount to unravelling the complicated relationship between the microbes that colonize the vaginal mucosa, mucosal immune cells and HIV transmission.

For many years the diagnosis of BV is made clinically using Amsel's criteria,[5] or microscopically by means of Nugent's scoring system,[6]. The perceived role of *Gardnerella vaginalis* in the aetiology of BV has varied over the years. After its first description in the early 1955,[7] it was thought to be the single causative agent of BV. Later, its role was questioned since the organism was also found in women without this condition. More recently molecular techniques identified a diverse group of bacteria associated with BV,[8-9]

Bacterial vaginosis is a condition in which the vaginal microbiome is altered. The immune response of which is seen as vaginal inflammation and the presence of vaginal discharge. However, some patients remain asymptomatic. The vaginal flora in healthy women has an abundance of *Lactobacillus* that produce L-lactic acid which inhibits many other microbial species,[10]. Lactic acid production also decreases HIV transmission by trapping the virus in acidic mucus,[11]. BV is characterized by a decreased concentration of lactobacillus in the vagina and an increase of diverse microbial species. However, the cause of BV still remains unclear. Recent publications have identified a distinctly different vaginal microbiome in South African women where *Lactobacillus* species dominate only in a minority of asymptomatic individuals,[12].

Microscopy is the hallmark for laboratory diagnosis of bacterial vaginosis. Our study aimed to identify by microscopic observation, differences in bacterial morphotypes in patients presenting with vaginal discharge syndrome. Using molecular techniques we determined the relative quantities of the microscopically most frequently observed bacteria. We also measured the effect of these genera on the vaginal immune response.

METHODOLOGY

Specimen collection and preparation

One hundred and sixty one consenting patients of 18 years and older presenting with vaginal discharge were recruited at a primary healthcare clinic in Umlazi-Durban, South Africa. Patients were treated syndromically and no follow up was done. Diagnostic testing of common STIs were performed. Patients with a genital ulcer were tested for the etiological agents of this syndrome. Those positive were excluded. A total of 122 tested negative for *Chlamydia*

trachomatis, *Neisseria gonorrhea* and *Trichomonas vaginalis*. These 122 patients were included in this study.

Specimens were collected from all 161 women recruited.

A standard dacron swab on a rigid shaft was inserted approximately 5 cm into the vagina and withdrawn while rotating. Once removed the swab was rolled onto a glass slide to prepare a smear for microscopic analysis.

A vaginal applicator tampon (Tampax® Regular) was inserted into the vagina, removed after approximately 1 minute and placed in 10 ml phosphate buffered saline (PBS, pH 7.2) for transport [13].

Upon arrival in the laboratory, the tampon fluid was expressed using an autoclaved wooden tongue depressor. The average yield was 6.5 ml. One ml of this was stored at -20ºC for DNA isolation. The remaining fluid was centrifuged at 1600 rpm for 10 min. The supernatant was removed and the pellet was re-suspended in the remaining 1 ml of fluid and prepared for flow cytometry.

Microscopy

The vaginal smear was gram stained, viewed under light microscopy at 1000x magnification and scored using Nugent's criteria, [6]. A blinded reading of all slides was performed by 2 trained laboratory personnel. Patients were classified based on the dominant bacterial morphotype.

Flow cytometry

Each cell suspension was split into four 250 µl aliquots. Each aliquot was centrifuged at 1600 rpm for 10 min. The supernatant was removed and the cell pellet of three of the four aliquots was stained with one of the panels of fluorochrome conjugated antibodies (Table 1). The fourth pellet was used as a control for auto-fluorescence. Samples were run on the BD ***FACSCanto™ II (BD Biosciences)***. Analysis was performed using FlowJo V10 software (Treestar®).

Table 1: Cell markers used for flow cytometry

Panel 1	
Marker	**Population**
CD3	T cells
CD14	Macrophages
CD11b	Monocytes
CD11c	Dendritic Cells (all DC subsets express this marker)
CD207	Langerhans Cells (skin resident DCs)
CD209	Non-LH dendritic cells (DC-SIGN carriers)
Panel 2	
Marker	**Population**
CD3	T cells
CD11b	Monocytes
CD11c	Dendritic Cells (all DC subsets)
CD19	B cells
CD80	co-stimulatory molecule expressed on activated B-cells, DCs and monocytes
CD86	co-stimulatory molecule expressed on activated B-cells, DCs and monocytes
Panel 3	
Marker	**Population**
CD3	T cells
CD4	T helper cells
CD8	Cytotoxic T cell
CD19	B cells
CD16	NK cells
CD56	NK cells

Real Time Quantitative PCR (QPCR)

Lactobacillus jensenii, (ATCC®25258™), *Gardnerella vaginalis*, (ATCC®14018 ™), *Veillonella parvula*, (ATCC®17745 ™) and *Prevotella intermedia*, (ATCC®25611™) were used as representatives of the morphotypes that were most frequently observed. These reference strains were grown on suitable media and the yield was suspended in 1 ml of PBS, pH 7.2, to obtain a turbidity of OD 0.5 (600 nm),[14].

DNA was extracted from 1 mL of tampon fluid from each patient as well as from the reference strain suspensions. Fluids and suspensions were centrifuged at 3000g and the deposits were re-suspended in 200µl PBS, pH 7.2. This was followed by extraction using the QiaAmp DNA mini kit, (Qiagen).

QPCR was performed on the ABI 7500 Real Time PCR system using the Sybre green absolute quantitation program. Sequences of the genus specific primers and the annealing conditions were taken from previously published studies,[14–16]. The Fast start essential DNA Green Master (Roche Diagnostics) was used to prepare the mastermix as per manufacturer's guidelines. The relative percentage of each bacterial type was determined for each sample.

Statistical Analysis

Medians and interquartile ranges (iqr) were used to summarize bacterial load. The non-parametric Wilcoxon signed rank test was used to compare bacterial loads done on the same woman. The Kruskal-Wallis test was used in the analysis of immune marker distribution between the three Nugent's defined groups with the Bonferonni adjustment for pairwise comparisons. Statistical analysis was performed using STATA version 13.1.

RESULTS

Quantitative bacteriology

The relative quantities of the four genera of bacteria tested are shown in figure 1 for each of the 3 Nugent score groups.

Lactobacillus spp. dominated in the group with 0 to 3 scores. The group scoring 7 to 10 was dominated by *G. vaginalis*. The relative quantities of both bacterial types were significantly higher than that of the other three genera in their respective groups, (Fig. 1). The intermediate group (score 4 to 6) had elevated levels of all genera when compared with the 0-3 group, except for *Lactobacillus* spp. However, the differences observed did not reach statistical significance.

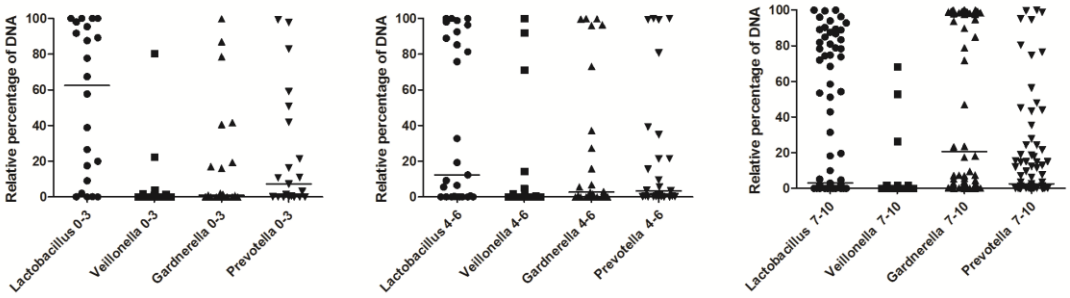

Figure 1: Relative percentage of microbial DNA associated with BV in each of the Nugent's groups

The relative percentage of genus specific DNA is plotted for each genus tested in the different Nugent's score groups. The median percentage is shown as the horizontal line. Nugent score 0-3:*Lactobacillus* spp compared to *Veillonella* spp (p=0.0002), *Gardnerella vaginalis*(p=0.02) and *Prevotella* spp (p=0.05). Nugent score 7-10: *Gardnerella vaginalis* compared to *Lactobacillus* spp (p=0.03), *Veillonella* spp (p<0.0001) and *Prevotella* spp. (p=0.007)

The relative bacterial load of two of the four bacterial genera showed an association with the Nugent score, (Fig. 2). The amount of *Lactobacillus* spp. decreased and that of *Gardnerella vaginalis* increased with increasing Nugent scores. Both trends were statistically significant.

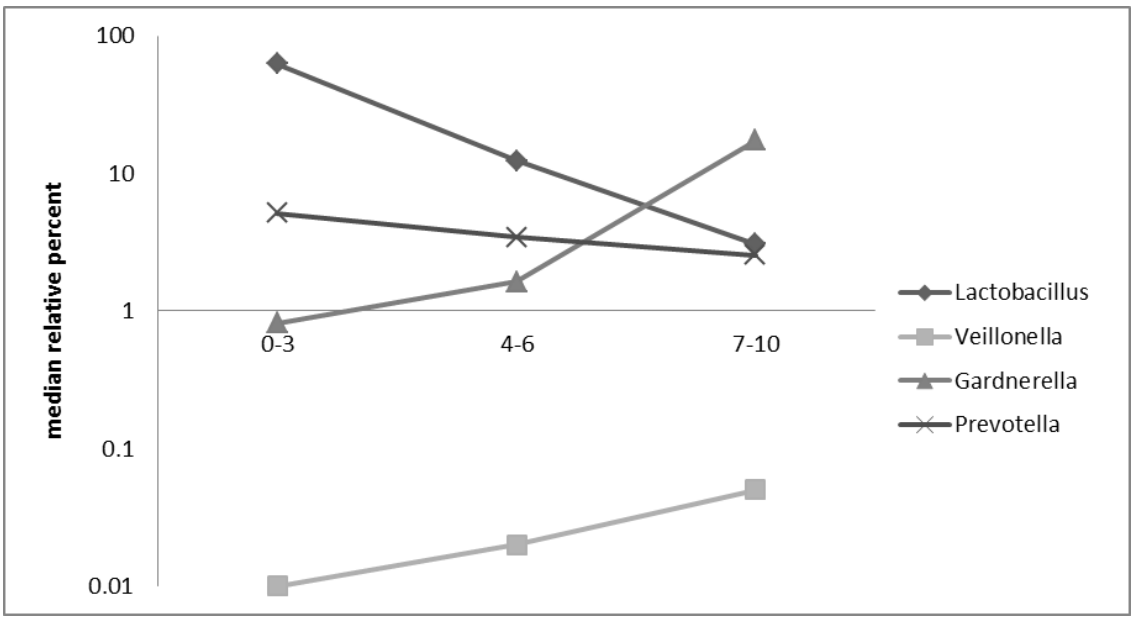

Figure 2: Bacterial profile across the Nugent's groups
The log of the relative median percentage is plotted for each genus in the different Nugent score groups. Spearman's correlation between Lactobacillus and Nugent score group 7-10 (r_s= -0.17), Gardnerella and Nugent score group 7-10 (r_s= 0.3) Veillonella and Nugent score group 7-10 (r_s= 0.02) and Prevotella and Nugent score group 7-10 (r_s= -0.05)

Association between vaginal immune cells, Nugent score and dominant bacterial genus

The distribution of the cell markers in the three groups according to the Nugent score are shown in Fig. 3 (a). The percentage of NK cells ($p<0.02$), Langerhans ($p<0.04$), non-LH dendritic cells (DC SIGN carrier)($p<0.02$), monocytes($p<0.003$) and activated macrophages ($p<0.009$) in the group with Nugent score 7-10 were significantly higher compared to the group with score 0-3.

Figure 3(b) shows the percentages of immune cells in patients grouped according to dominant bacterial genus. Dominance was defined as the highest relative percentage observed by PCR in each patient. The group dominated by *G. vaginalis* had a significantly higher percentage of activated B cells ($p<0.005$), NK cells ($p<0.0001$), Langerhans cells ($p<0.0001$), non-LH dendritic cells (DC SIGN carrier)($p<0.0001$) and macrophages ($p<0.003$) when compared to the *Lactobacillus* spp. dominated group.

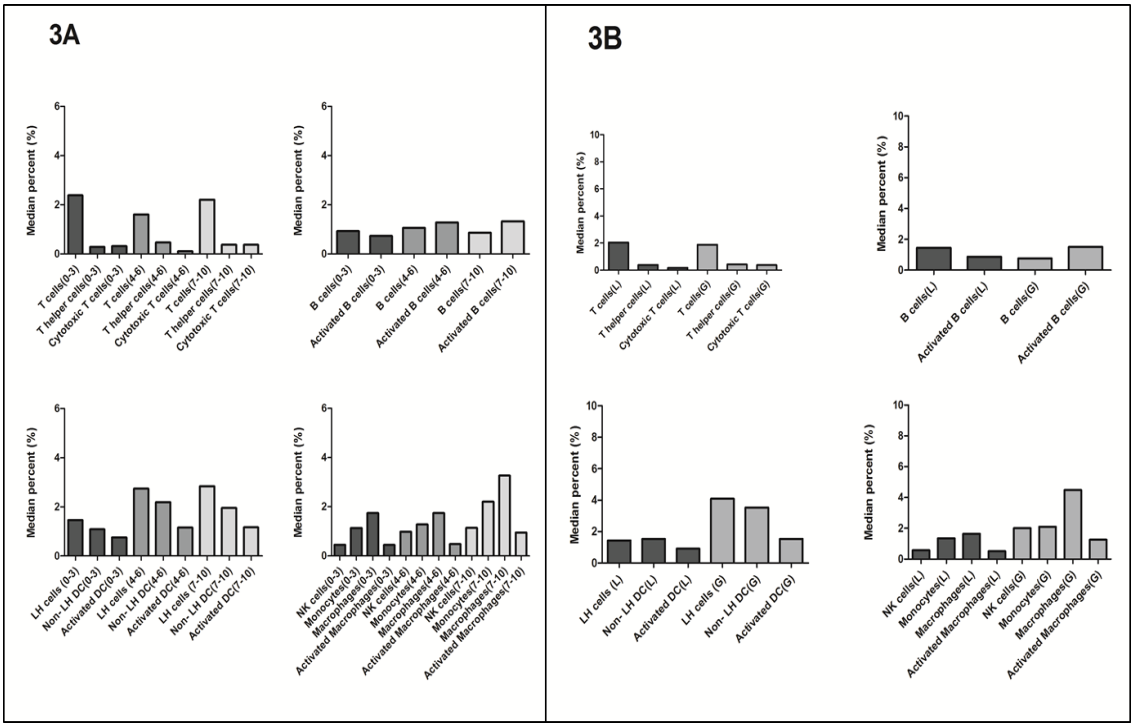

Figure 3: Immune response of patients within the Nugent's group
The bars represent the median percentage of each immune cell population tested. 3A shows the immune cell populations by Nugent score group and 3B by dominant bacterial genus. L = Lactobacillus; G = Gardnerella

DISCUSSION

Our study population consisted of women with abnormal vaginal discharge in which no known STI etiology could be established with our panel of PCRs. In this group we looked for correlations between Nugent score, dominant bacterial genus and mucosal immune cell populations. *Mycoplasma genitalium* was not part of our PCR panel. Therefore some women may have had an infection with this organism.

In keeping with the literature we found *Lactobacillus* spp. to be the dominating bacterial type in the group with the lowest Nugent score. Originally, *G. vaginalis* was thought to be the causative agent of BV. With advancement in molecular detection techniques, several other bacterial species were proposed as possible etiological agents. The putative role of *G. vaginalis* was thus diminished. Our cohort of symptomatic women in the group with Nugent score 7 to 10 was found to be dominated by *G. vaginalis*. The relative amount of *Prevotella* spp. remained constant throughout the Nugent score. The relative amount of *G. vaginalis* showed a significant upward trend with increasing Nugent score, (p=0.0007). *Veilonella* spp. showed a trend similar to *G. vaginalis* but at a much lower level (Fig 2).

In the Nugent score group 4-6 the relative concentration of *G.vaginalis* starts to change from second lowest to highest when score 7-10 is reached. This correlates with a significant drop of

the relative concentration of *Lactobacillus* spp. from Nugent score 0-3 to Nugent score 4-6. The decline in levels of inhibitory factors associated with *Lactobacillus* such as maintenance of a low pH by production of lactic acid and the production of H_2O_2[17] may provide an opportunistic environment for multiplication of other bacterial species. *G. vaginalis* seems to make better use of this changing environment than *Prevotella* spp. or *Veillonella* spp. Another option is that the exponential increase in *G. vaginalis* concentration drives the change in Nugent score and the depletion of *Lactobacillus* spp.

Several authors postulate a synergistic relationship between *G. vaginalis* and the multiple anaerobic species present in the vagina during BV,[18-19]. One study showed that *Prevotella bivia* produces ammonia in peptone enriched VDM (vaginal defined medium) which was used by *G. vaginalis* for the production of amino-acids. They postulated that these amino-acids stimulated the growth of other anaerobic species [19]. We did not find an increase of *Prevotella* spp. with increase Nugent score. However, the relative percentage of *Prevotella* spp. remained constantly high which may be in keeping with the provision of ammonia to stimulate growth of *G. vaginalis*. The increase in *Veillonella* spp. relative percentage which starts at mid-level Nugent scores could be the result of growth stimulation due to amino-acid production by *G. vaginalis*. This interplay between species may be responsible for increase in pH with subsequent inhibition of *Lactobacillus* spp.

Despite the frequent microscopic observation of *Veillonella* morphotypes, at the DNA level *Veillonella* species was present at the lowest levels of the four species tested. This suggests that the gram negative cocci observed belonged to different species. While there is no recognised causative agent for BV it is agreed that this condition is characterised by the presence of a polymicrobial microbiome. While it is generally accepted that predominance of lactobacilli is compatible with a healthy vaginal environment, studies on the vaginal microbiome of South African women without abnormal vaginal discharge showed that *Lactobacillus* spp. is not always dominant in the vagina of 'healthy' patients,[12]. Additionally, those that had lactobacillus dominance, differ by species with either *Lactobacillus crispatus* or *Lactobacillus iners*,[20]. The differences in the dominant bacterial type of a 'healthy vaginal state' seen in South African women are reflected in the commensal bacteria present. Table 2 shows a comparison of bacterial colonization between this study in symptomatic women and the study by Anahtar *et al*,[12]. The only statistically significant difference between symptomatic and asymptomatic women was a higher percentage with mixed flora in the asymptomatic group. This is likely based on differences in study design. Application of next generation sequencing in the symptomatic group would possibly have increased the number of women with mixed flora.

Table 02: Comparison of bacterial load between asymptomatic and symptomatic patients
The study cohort of this study was grouped to match data accordingly. Lactobacillus dominance = CT1+2, Gardnerella dominance = CT3 and mixed species = CT4

	Number (%) with		
	Anahtar *et al*	this study	*p*-value
Lactobacillus dominance	54 (37%)	66 (41%)	0.5
Gardnerella dominance	41 (28%)	59 (37%)	0.1
mixed species	51 (35%)	36 (22%)	0.02
Nugent score 7-10	73 (50%)	79 (49%)	0.9

The vaginal immune response to bacteria and the ability to initiate an inflammatory response or induce chemotaxis has major importance in disease and health. A number of studies have shown that *Lactobacillus* associated with a healthy vagina or lactic acid produced has little or no effect on inflammatory response,[21]. Contrary to this, bacteria associated with BV and short chain fatty acids produced by these anaerobes do stimulate an inflammatory response,[22]. We found that increase in Langerhans cells; non-LH dendritic cells and macrophages were associated with dominance of gardnerella. The Anahtar study,[12] in asymptomatic patients found in the gardnerella dominant group a trend towards increased cytokine levels that are produced by the cells that we found significantly elevated in symptomatic women with gardnerella dominance. This suggests that *G. vaginalis* initiates an immune response the level of which determines whether a women is symptomatic or not. This is supported by our finding that there is a significantly higher expression of NK cells, Langerhans cells, non-LH dendritic cells, monocytes and activated macrophages in the BV group compared to the group with the lowest Nugent score. The latter was dominated by *Lactobacillus* spp. while the BV group was dominated by *G. vaginalis*.

A recent in vitro study showed a dose dependent relationship between *G. vaginalis* and stimulation of an immune response. This resulted in maturation of dendritic cells and reduction in internalization capacity,[23]. Characteristics associated with virulence of *G. vaginalis* are the ability to form biofilms,[24]and the production of a cytolysin. It has been postulated that its virulence is increased by synergistic interaction with other BV associated bacteria. An example of this is the sialidase of *Prevotella* species and *Bacteroides* species that break down mucus and so allowing better adhesion of *G. vaginalis* to the vaginal epithelium. High vaginal levels of sialidase also prevented an IgA response to *G. vaginalis* cytotoxin, thereby mitigating the immune response, [25].

There is an association of increased HIV expression and transmission in BV patients,[1]. Our study shows a significantly higher number of HIV target cells (Langerhans, DC-sign and macrophages) in the BV group and the group dominated by *Gardnerella* spp. Langerhans cells infected with HIV have the ability to travel to the lymph nodes disseminating the virus. The observation of a significantly higher number of monocytes in the BV group shows the potential for BV associated stimuli to promote differentiation to macrophages or dentritic cells. We show that populations dominated by *Gardnerella vaginalis* have an increased expression of macrophages when compared to *Lactobacillus* spp. dominant groups.

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Conflicts of Interest: No conflict of interest declared.

Ethical Statement: The work reported in this manuscript has been approved by the ethics committee of the University of KwaZulu-Natal. Informed consent was received for all patients.

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CHAPTER FOUR

Manuscript entitled:

"Increased shedding of HIV and HSV-2 in patients with bacterial vaginosis is associated with high bacterial load of *Gardnerella vaginalis*"

Submitted to:

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MOI:

Increased shedding of HIV and HSV-2 in patients with bacterial vaginosis is associated with high bacterial load of *Gardnerella vaginalis*

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number of: references(21), figures(2) and tables(1)

Summary

Increased concentrations of *Gardnerella vaginalis* was associated with increased vaginal shedding of HIV and HSV-2 in symptomatic women with no detectable STI.

ABSTRACT:

Background

Detection of vaginal HSV-2 shedding has been associated with increased vaginal HIV RNA copies. Bacterial vaginosis (BV) is implicated in the transmission of HIV. Patients with BV have been shown to increase the frequency of HSV-2 shedding.

Methods

Consenting symptomatic female patients >18 years were recruited from a primary healthcare clinic (PHC) in Durban. Serological tests to detect HSV-2 and HIV were performed. Vaginal bacterial concentrations and HIV viral loads were determined by QPCR. Conventional PCR was done to ascertain which patients were shedding HSV-2 at the time of collection.

Results

Lactobacillus spp. was associated with the group that was HIV antibody negative and *Prevotella* spp with the HIV antibody positive group ($p < 0.05$). *Prevotella* spp. was not associated with shedding of HIV. The bacterial copies of *Gardnerella vaginalis* was higher in patients shedding HSV-2 and significantly higher copies in those shedding HIV, ($p < 0.05$).

Conclusions

This study emphasizes the role of BV in the transmission of HIV. We show that *G. vaginalis* and not *Prevotella* spp. is associated with HSV-2 and HIV shedding.

Key Words: Bacterial vaginosis, HSV-2, *Gardnerella vaginalis*, HIV

INTRODUCTION

Bacterial vaginosis (BV) is associated with HIV infection. Several studies have shown that mixed vaginal bacterial populations result in higher concentration of HIV 1 RNA in the endocervix.[1,2] In vitro studies have shown that the cervicovaginal mucus from Lactobacillus dominant women have a protective effect against HIV infection.[3] During BV there is increased diversity of anaerobic bacterial species. Succinic acid, a metabolic product of a variety of anaerobic bacterial species associated with BV, was found to increase HIV expression in HIV infected monocyte derived macrophages.[4]

Upregulation of pro-inflammatory cytokines has been reported in women with high Nugent scores irrespective of the presence of symptoms associated with BV. In the same study, it was found that cytokine concentrations were much lower during HSV shedding and *Trichomonas vaginalis* infection.[5] HSV 2 infection and in particular vaginal shedding of HSV, is associated with both the acquisition and shedding of HIV RNA.[6–8] A Gram stain score compatible with bacterial vaginosis was found to increase the frequency of HSV shedding in HSV-2 seropositive women.[9] A bacterial microbiome associated with BV elicits a pro inflammatory response which is capable of disrupting the epithelial barrier function.[10] This increases the risk of infection with bacterial STIs, HSV-2 and HIV.

This study aimed at exploring the association of the bacterial morphotypes most frequently observed during microscopic diagnosis of BV with the shedding of HSV and HIV.

METHODOLOGY

Consecutive consenting patients presenting with vaginal discharge were recruited on weekdays between 8:00 am and 12:00 from October till December 2014 at the Umlazi D clinic in Durban, KwaZulu-Natal, South Africa. Ethical approval was obtained from the Biomedical Research Ethics Committee – University of KwaZulu-Natal.

A Dacron swab was inserted for approximately 5 cm into the vagina and withdrawn while rotating. Following this, a vaginal tampon (Tampax® regular) was inserted and left for 1 minute. On removal the tampon was placed into 10 mL PBS (pH7.0).[11] Approximately 4 ml of clotted blood was collected by venapuncture.

Smears for microscopy were prepared by rolling the vaginal swab onto a glass slide followed by Gram staining. Bacterial colonization was assessed using Nugent's score criteria.

Upon arrival in the laboratory, the tampon fluid was expressed using an autoclaved wooden tongue depressor. The average yield was 6.5 ml. One ml of this was stored at -20ºC for DNA isolation. The remaining fluid was centrifuged at 500g for 10 min. The supernatant was removed and stored at -20ºC for HIV quantitative PCR.

Clotted blood was centrifuged at 1500g for 10 min following which the serum was used for HIV antibody testing with the one step HIV rapid kit (CORE™, Beijing, China) as per manufacturer's recommendations. HIV antibody tests were performed on tampon fluid of those patients that refused venipuncture.

Lactobacillus jensenii, (ATCC®25258™), *Gardnerella vaginalis*, (ATCC®14018 ™), *Veillonella parvula*, (ATCC®17745 ™) and *Prevotella intermedia*, (ATCC®25611™) were used as representatives of the morphotypes that were most frequently observed. All reference strains except *Gardnerella vaginalis* were grown on brain heart infusion (BHI) agar supplemented with 5% yeast extract, 5mg/l heamin and 1mg/l menadione. *Gardnerella vaginalis* was grown on BHI supplemented with 10% heat inactivated horse serum. The yield was suspended in 1 ml of PBS, pH 7.2, to obtain a turbidity of OD 0.5 (600 nm).[12]

DNA was extracted from 1 mL of tampon fluid from each patient as well as from the reference strain suspensions. Fluids and suspensions were centrifuged at 3000g and the deposits were re-suspended in 200 µl PBS, pH 7.2. This was followed by extraction using the QiaAmp DNA mini kit, (Qiagen, Hilden, Germany).

QPCR was performed on the ABI 7500 Real Time PCR system using the Sybre green absolute quantitation program. Sequences of the genus specific primers and the annealing conditions were taken from previously published studies,[12–14]. The Fast start essential DNA Green Master (Roche Diagnostics, Indianapolis, USA) was used to prepare the mastermix as per manufacturer's guidelines. The relative percentage of each bacterial type was determined for each sample.

A conventional PCR to detect the presence of HSV-2 DNA was performed on the ABI 9700 thermal cycler. Primer sequences and cycling conditions were as previously published.[15]

Two milliliters of the cell free supernatant was centrifuged at 15 000 rpm for 3 hours at 4ºC. Most of the supernatant was discarded and the pellet was re-suspended in the remaining 140 µl. The Qiamp viral RNA extraction kit (Qiagen, Hilden, Germany) was employed as per manufacture's guideline resulting in a final elution volume of 100µl. The commercially available HIV Real-TM Quant kit (Sacace Biotechnologies, Lombardia, Italy) for the quantitative detection of HIV was used.

QPCR was performed on the ABI 7500 Real Time PCR instrument. Standards were run in duplicate for each run. Internal controls (IC) contained in the kit were added to each sample prior to extraction. This controlled for loss during extraction and inhibition of PCR in the calculation of viral quantity. The concentration of HIV RNA was calculated using the given formula:

$$\frac{\text{HIV DNA copies/specimen}}{\text{IC DNA copies/specimen}} \times \text{coefficient} = \text{copies HIV/mL}$$

The coefficient is specific for each lot and is reported on the data card within the kit.

Statistical Analysis

Medians and interquartile ranges (iqr) were used to summarize HIV and bacterial loads. The non-parametric Wilcoxon signed rank test was used to compare viral and bacterial loads done on the same woman. Statistical analysis was performed using STATA version 13.1.

RESULTS

A total of 161 patients were recruited of which 79 patients were found to be positive for HIV antibodies, 30 on serum and 49 on vaginal fluid. Sixteen (20%) of these 79 patients were shedding HSV-2 while the remaining 63 did not shed this virus at the time of collection. Vaginal shedding of HIV was detected in 10 and 32 HSV-2 shedders and non-shedders respectively. The median vaginal HIV viral load in the group that shed HSV-2 was 11650 copies/ml and in the HSV-2 non-shedders this was 8670 copies/ml (p=0.0959), (Fig 1).

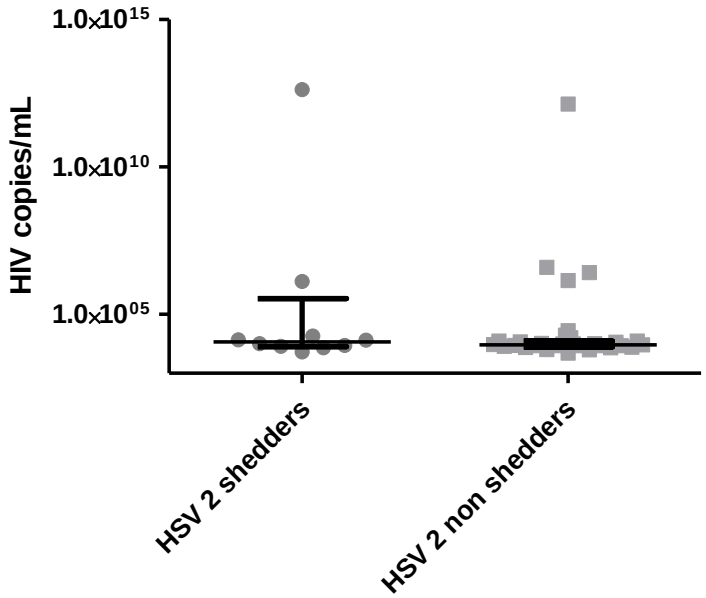

Figure 1: Vaginal HIV viral load in patients simultaneously shedding HSV-2 and in patients who were not shedding HSV-2. Median and interquartile range are represented graphically

Of the 161 patients enrolled, 122 had no detectable sexually transmitted infections other than BV. On Gram stain microscopy four bacterial morphotypes were most frequently observed. QPCR for the genera representing these four morphotypes were performed on tampon fluids of all women enrolled. In the 122 without STIs, *Lactobacillus* species was found to have the highest relative concentration in the group with Nugent score 0-3 while the percentage of *Gardnerella vaginalis* was highest in the group with Nugent score 7-10. The 4-6 group had an almost even mix of the bacterial genera tested with no statistical differences observed between genera except for *Veillonella* spp. which relative percentage was statistically lower than that of the other three, (Fig 2). Of the 122 women 39 shed HIV.

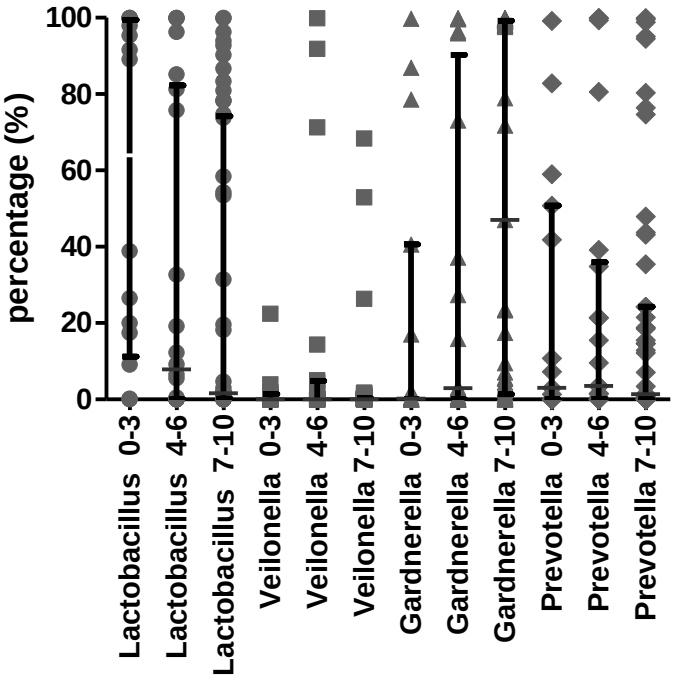

Figure 2: Aligned dot plot showing the relative percentage of 4 defined vaginal bacteria stratified by Nugent's score groups. Median and interquartile range are represented graphically.

There was a trend to higher relative concentrations of *Prevotella* spp. in the HIV antibody positive group while more *Lactobacillus* spp. were found in the HIV antibody negative group. However, none of these differences reached statistical significance (Table 1). In the 56 HIV antibody positive patients, the relative concentration of *Gardnerella vaginalis* was highest in women that also shed HIV in the vaginal secretions (p=0.02). Contrary to this *Prevotella* spp. was significantly associated with non HIV shedding, (p=0.02) (table 1). There was a greater median bacterial load of *Lactobacillus* spp. *Veilllonella* spp. and *Gardnerella* spp. in the group that shed HSV-2 in the vaginal secretions compared to the group that did not, (table 1). The latter observation is the same for the 66 HIV antibody negative patients, except for *Veillonella* spp. which remained constant.

Table 1: Relative concentration of bacteria in patients shedding HIV and HSV-2
Median percentage of bacterial genus represented with (iqr). Significance observed at $p \leq 0.05$

	Median (iqr)			
	Lactobacillus	Veillonella	Prevotella	Gardnerella
HIV antibody negative (n=66)	29.02 (89.9)	0.02 (0.26)	1.34 (15.53)	9.51 (95.86)
HIV antibody positive (n=56)	4.13 (78.41)	0.05 (0.60)	8.51 (40.4)	7.1 (97.4)
HIV shedders (n=29)	0.759 (78.06)	0.04 (0.4)	3.02 (14.5)	83.5 (98.2)
HIV non-shedders (n=27)	17.42 (78.7)	0.084 (0.977)	21.35 (92.60)	2.244 (27.02)
HSV-2 shedder (n=12)	28.18 (84.31)	0.07 (0.77)	1.55 (94.0)	10.75 (36.25)
HSV-2 non-shedder (n=44)	2.52 (78.22)	0.05 (0.60)	7.48 (97.44)	7.39 (40.31)
p-value HIV antibody positive vs HIV antibody negative	0.053	0.8	0.03	0.84
p-value HIV shedders vs HIV non shedders	0.7	0.2	0.02	0.02
p-value HSV-2 shedders vs HSV-2 non shedders	0.5	0.7	0.4	0.9

In the group that tested positive for sexually transmitted infections (*Neisseria gonorrhoea, Trichomonas vaginalis, Chlamydia trachomatis, Treponema pallidum and Haemophilus ducreyii*), *Prevotella* spp. had the highest relative concentration in the group with Nugent's score 0-3. However, no significant differences were observed between the Nugent score groups. *Lactobacillus* spp. was highest in women with Nugent score 4-6, while *Gardnerella vaginalis* was highest in those with Nugent score 7-10. Although statistical differences were observed between *Lactobacillus* spp. and *Veillonella* spp. in the Nugent score group 4-6 and between *Gardnerella* and *Prevotella/Veillonella* in Nugent score group 7-10 , none of the bacteria dominated within any of the Nugent's score groups.

DISCUSSION

Several reports link shedding of HSV-2 with increased HIV transmission.[16,17] It has been shown that increased levels of cervical HSV-2 shedding were associated with the presence of increased cervical HIV RNA concentrations.[18] In keeping with the literature we report a higher HIV viral load in patients shedding HSV-2 compared to those that were not. Failure to reach statistical significance is likely because analysis was limited to the 79 HIV positive patients.

BV has been implicated as a risk factor for the transmission of HIV. This is thought to be a result of changes in pH brought on by disruption of the H_2O_2 producing *Lactobacillus* dominant

environment seen in healthy women.[10] We show that the intermediate group (score 4-6) and the BV group (score 7-10) had higher median number of HIV copies than the group with score 0-3. The intermediate group had the highest vaginal HIV viral load though this did not reach statistical significance. Molecular analysis of 4 bacterial genera confirms the presence of a Lactobacillus dominant community in the women with a 0-3 Nugent score and was associated with the lowest level of HIV shedding. The intermediate group showed a drastic decline in the relative percentage of *Lactobacillus* spp. allowing for the increased bacterial diversity. In addition there were no statistical differences in relative percentages between the genera in the intermediate group except for *Veillonella* spp. which was lower than the other genera. A limitation of our approach is that we tested for only four genera. Including more species could change this picture.

Individuals infected with HIV have enteropathy with increased inflammation.[19] Gut flora rich in *Prevotella* spp. has been associated with HIV infected individuals.[20] Another study on South African females reported the highest vaginal inflammatory response in patients with greater than 1% *Prevotella bivia* which increases inflammation by the release of lipopolysaccharide (LPS). This inflammatory response increases the likelihood of becoming infected with HIV.[21] In this study *Veillonella* spp. and *Prevotell*a spp. had higher relative percentage in the HIV antibody positive group with *Prevotella* spp. reaching statistical significance.

Within the HIV antibody positive group, *Gardnerella vaginalis* and *Veillonella* spp. were associated with the group that shed HSV-2. Previous studies have reported an association between cervico-vaginal shedding of HSV-2 with increased genital HIV RNA and DNA.[17]

We report a negative association between *Prevotella* spp. and HIV shedding while *Gardnerella vaginalis* showed a positive association. Additional logistic regression based on grouping bacteria by dominance (*Lactobacillus* spp. *Gardnerella vaginalis* and mixed species (*Veillonella* and *Prevotella*)), the odds of HIV shedding was 4.5X greater in the group dominated by *Gardnerella vaginalis* (OR: 4.5: 95%CI 1.13-17.99) when compared to Lactobacillus dominance. In our study population, vaginal environments dominated by *Gardnerella vaginalis* were found to have significantly elevated levels of immune cells. . These included macrophages, Langerhans cells and non- Langerhans denditric cells (DC-SIGN positive) which are targets for HIV infection.

This study indicates a role of BV in the transmission of HIV and HSV-2 and we show that this is related to the concentration of *G. vaginalis* in the vaginal content but not to the concentration of species belonging to the genus *Prevotella*.

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CHAPTER FIVE

DISCUSSION AND CONCLUSION

In this study patients were recruited from two different PHCs in KZN. One of which is situated near a taxi rank close to the central business district in the capital city of KZN and the other in a township away from the Durban CBD. We found the prevalence of vaginal discharge pathogens significantly higher in the clinic close to the taxi rank as opposed to the township. Two of the three pathogens cause acute infections and the accompanying symptoms of the disease point to the presence of a STI and relate to sexual behavior. Therefore patients are likely to travel to PHCs away from their residence for treatment. The clinic in the township had more patients with BV and HSV, which are more chronic conditions.

Of the patients with ulcers, HSV-2 was observed in 4 of 13 patients and 1 patient was diagnosed with LGV. However, 69% (9/13) had no detectable aetiology. In 1991 the assessment of genital ulcers in women reported a higher frequency of ulcers of which bacterial aetiology was the main cause. HSV was detected in 18% while another 18% had no aetiology,(O'Farrell et al. 1991). While we report a fewer number of ulcers, the majority remains without aetiology and HSV-2 remains the most detected pathogen. The study by Leichliter et al, (2016), in South African men, found of the ulcers that had detectable aetiology, HSV -2 was the most prevalent (71.4%). Bacterial aetiology was found less frequently, in 20.8% there was no pathogen identified,(Leichliter et al. 2016). We observed fewer ulcers in our population; this is due to the recruitment of women with a discharge of which ulcers were a secondary observation.

In both clinics BV had the highest prevalence; this was followed by *T. vaginalis* infection. This finding is supported by the study of Mlisana et al. 2012 in high risk women in Durban, KZN. The combined prevalence of *N. gonorrhoeae* from the Boomstreet and Umlazi clinic was 14.2% (93/625). We report a prevalence range from 3% to 12% in the studies described in table 2 of the manuscript titled: "Prevalence of sexually transmitted diseases in women in KwaZulu-Natal". There is an increase in the number *N. gonorrhoeae* infections but this is not significant. However, our study was in symptomatic patients and most of the studies quoted in table 2 were not. While there are no observed differences in prevalence for *C. trachomatis* and *T. vaginalis*, prevalence of BV has increased.

Gardnerella vaginalis plays a role in the pathogenesis of BV

We found in women with vaginal discharge syndrome that the prevalence of BV was higher than that of infection with any of the STI pathogens. The causative agent of BV has to date not been established. The condition is described as a polymicrobial state in which there is a reduction of *Lactobacillus* spp. and an increase in anaerobic bacteria,(Fredricks et al. 2005). A vaginal microbiome that is dominated by *Lactobacillus* spp. is thought to prevent HIV and many urogenital infections including bacterial vaginosis and STIs by the production of lactic acid which lowers the vaginal pH, (Lai et al. 2009; Wiesenfeld et al. 2003). Although in 1955

G. vaginalis was thought to be the etiological agent of BV, the discovery of unculturable microbes associated with the condition have identified these as possible pathogens, (Gardner & Dukes 1955; Fredricks et al. 2005). This was further supported by the isolation of *G. vaginalis* from women without BV,(Aroutcheva et al. 2001). However, this holds true for recognized STI pathogens since all of these do cause asymptomatic infections.

Microscopy still remains the most commonly used method for laboratory diagnosis of BV. We quantified 4 of the most frequently observed morphotypes by QPCR. In keeping with the literature we found that *Lactobacillus* spp. dominated the group with low Nugent score while *G. vaginalis* dominated the group with high Nugent scores. Although *G. vaginalis* was also detected in the women with lower Nugent scores, the quantity detected increased with increasing Nugent score. This trend was also observed with the genus *Veillonella* spp. but the increase in number of copies did not reach statistical significance. *Prevotella* spp. remained relatively constant in all three Nugent score groups. Bradshaw et al, (2006), reported an increasing concentration of *Atopobium vaginae* with rising Nugent score with a significantly higher copy number detected in the 7-10 group. They describe the same trend for *G. vaginalis*. *A. vaginae* was found only in combination with *G. vaginalis*. The latter statement supports the role of *G. vaginalis* in stimulating the growth of other bacterial species associated with bacterial vaginosis.

Prevotella bivia has been reported to support the growth of *G. vaginalis* and *Peptostreptococcus anaerobium* by production of ammonia and amino acids, (Pybus & Onderdonk 1997; Pybus & Onderdonk 1998). The observation of symbiotic relationships between bacteria associated with BV supports the presence of a polymicrobial state as described in patients with BV. However, the question remains as to what drives the establishment of this polymicrobial state.

For the establishment of any infection it is necessary for sufficient adherence to host cells. Tissue culture experiments on cervical epithelial cells showed a greater adherence of *G. vaginalis* and *Peptoniphilus* spp. as compared to other anaerobes tested. *Veillonella* spp. and *Prevotella bivia* failed to adhere. In addition, *G. vaginalis* was capable of producing thicker biofilms and was the only species tested capable of cytotoxicity, (Patterson et al. 2010). In another in vitro study, *G. vaginalis* obtained from a patient with BV was able to displace *Lactobacillus crispatus* but not *Lactobacillus iners*. The strain of *G. vaginalis* obtained from a healthy patient had a minimal effect on both. *L. iners* also supported the adhesion capacity of the BV associated *G. vaginalis* strain, (Castro et al. 2013). The previous role of *G. vaginalis* as a pathogen in the condition of BV has been questioned after the advent of techniques capable of identifying unculturable bacteria. With every report on increased concentrations of the different anaerobes in patients with BV, the importance of *G. vaginalis* in this condition diminished. This study re-examines the role of *G. vaginalis*, in symptomatic women without the complication of an STI. We find that *G. vaginalis* is the most likely pathogen responsible for the condition of BV in this cohort of patients.

There are distinctly different strains of *G. vaginalis*, one which remains commensal and is detected in healthy patients and one associated with BV. Recurrence of BV is frequently reported. Harwich et al, (2010), reported on the resistance of *G. vaginalis* to several antimicrobials and the presence of several genes and proteins which promote antimicrobial

resistance. The transfer of resistance genes between bacterial species is well documented, (Ochman et al. 2000). This further underscores the importance of BV and treatment thereof in a population with a high incidence of STIs and HIV.

South Africa is plagued with a high prevalence and incidence of HIV. STIs and BV are associated with increased HIV transmission. We found in our study a significantly higher number of immune cells which serve as HIV targets in the group with BV and the group dominated by *G. vaginalis*. The group with BV had overall significantly higher copy numbers of *G. vaginalis* compared to the other bacteria tested. While the importance of *G. vaginalis* in the transmission of HIV is highlighted, the limitation of this study remains that only 4 genera of vaginal bacteria were analysed. The overall sero-prevalence of HSV-2 in our study was 73. In a subset of patients with no aetiological diagnosis other than BV for symptomatic vaginal discharge, the HIV viral load was higher in those patients that shed HSV-2. This is in keeping with McClelland et al, (2002), who showed an association between increased HIV RNA and increased HSV copies. *G. vaginalis* and *Veillonella* spp. were associated with the group that shed HSV-2. Further *G. vaginalis* was associated with increased HIV shedding.

In conclusion, this study reflects the high burden of STIs and HIV in women presenting with vaginal discharge at 2 PHCs in urban KwaZulu-Natal. We attempted to correlate for the first time in symptomatic patients without STI aetiology and ulcer pathogens, the association of BV and BV pathogens to vaginal immune response using immune cell differential identification. More recent publications on asymptomatic women from South Africa show that Prevotella spp. is present in patients with great bacterial diversity. We show a consistent presence of *Prevotella* spp. in all Nugent's groups. Further, we did not find any statistically significant increases in concentration of *Prevotella* spp. through the Nugent score groups indicating that's its role in the pathogensis of BV is more likely to alter the pH of the environment and by the production of metabolites that support the growth of other anaerobes more especially *G. vaginalis* which drives the condition of BV. We also report on an increasing concentration of *Veillonella* spp through the Nugent score group. Although the increase is not statistically significant, it adds to the establishment of the polymicrobial state of BV. This study highlights the importance of BV associated bacteria in the transmission of HIV. The initiation of a vaginal immune response and the ability to induce HSV-2 and HIV shedding emphasizes the role of *Gardnerella vaginalis* in vaginal pathology.

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APPENDICES

Staining for Flow Cytometry

Centrifuge 1.8ml of cell suspension at 1500rpm (4°C) for 10 minutes

Remove supernatant leaving 100ul behind

Add 1ul of each antibody according to panel design

Vortex and incubate at 2°C-8°C for 20 minutes

Add 500ul of FACS buffer (1% FBS in PBS)

Centrifuge at 1500rpm (4°C) for 10 minutes

Remove supernatant

Add 200ul fixative (4% paraformaldehyde in FACS buffer)

Culture media for ATCC strains

Lactobacillus, Veillonella and Prevotella

Brain heart infusion broth (BHI) supplemented with 5% yeast extract, hemin 5mg/L and vitamin K 1mg/L

Gardnerella vaginalis

BHI supplemented with 5% yeast extract and 10% heat inactivated horse serum