Investigating antimicrobial resistance patterns in *Gardnerella vaginalis* isolates

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DECLARATION

I, Thasmika Durga declare that;

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As the candidate's supervisor, I have read the thesis and have given my approval for submission for examination [redacted]

Supervisor: Prof Nathlee Abbai

Date: 20 November 2023

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ABSTRACT

Background: Bacterial vaginosis (BV) is gaining interest due to its problematic outcomes in women of reproductive age. Common symptoms are abnormal grey discharge that has a fishy odour accompanied by itchiness and pain. BV is characterized by the overall decrease in *Lactobacilli* and an increase in anaerobic facultative bacteria such as *Gardnerella vaginalis*. *G. vaginalis* is said to be the main species of bacteria involved in BV and is responsible for its pathogenicity. *G. vaginalis* has many virulence factors (formation of biofilms, mucus degrading sialidase and the production of the protein toxin, vaginolysin) that influence its pathogenicity, aiding in establishing infection. Treatment of BV includes the use of broad spectrum antibiotics such as metronidazole and clindamycin, which can be administered orally or in the form of an ointment. However, in clinical settings, antimicrobial resistance has increased due to resistant bacteria left behind after initial treatment, resulting in recurrent BV. The aim of the current study was to determine the susceptibility patterns of isolates of *G. vaginalis* to various antibiotics. This study also investigated the genetic diversity of isolates and linked genetic data with antimicrobial resistance patterns.

attending care at the antenatal clinic at the King Edward VIII hospital in Durban, KwaZulu-Natal, South Africa. A total of 150 pregnant women, 18 years and older, who were willing to provide written informed consent and willing to provide two self-collected vaginal swabs were enrolled in this study. Following sample collection, the first swab was placed in Amies transport media (Copan, Brescia, Italy) for the cultivation of *G. vaginalis*. The second swab was rolled onto a glass slide to diagnose BV by Nugent scoring on gram-stained vaginal smears. The genetic diversity assessments of the isolates were based on genetic differences in the *tuf* gene using clade specific primers on a quantitative polymerase chain reaction (PCR) platform. The antimicrobial susceptibility profiles were generated using the Sensititre™ Anaerobe MIC Plate (Thermo Fischer Scientific, United States).

Results: Sixteen isolates of *G. vaginalis* were cultured from pregnant women. Of the 16 isolates, five isolates were obtained from BV positive women, nine from BV intermediate women, and two were from BV negative women. The *16S rRNA* gene specific to *G. vaginalis* was amplified in all isolates, confirming the identity of the isolates. The genotyping/clade PCR revealed the presence of three clades. The frequencies for each clade were as follows; 100% for clade 1, 37.5% for clade 2 and 43.75% for clade 4. Multiple clades were found in 56.25% of the isolates. For antimicrobial susceptibility testing, only 14 isolates were viable for analysis. Of the 14 isolates that were successfully cultured and tested, 8/14 (57.1%) were susceptible to metronidazole (MIC of ≤8µg/ml) and 6/14 (42.9%) were resistant to metronidazole (MIC of ≥32µg/ml). Of the *G. vaginalis* isolates (n=5) cultured from BV positive women, 60% (3/5) of the isolates were susceptible to metronidazole whilst 40% (2/5) were resistant. There was no link between resistant patterns and infection symptoms, since one isolate was from an asymptomatic woman and the other isolate from a symptomatic woman. Of the *G. vaginalis* isolates (n=7) cultured from intermediate BV women, 43% (3/7) of the isolates were resistant to metronidazole while 57% (4/7) were susceptible. For intermediate women, there was a link between resistant patterns and infection symptoms, since all resistant isolates were cultured from symptomatic women. The *G. vaginalis* isolate cultured from the BV-negative women was susceptible to metronidazole and the woman was asymptomatic.

Of the six resistant isolates, five isolates (83%) were assigned to clade 1 alone. The remaining resistant isolate was a mixed clade, it was assigned clade 1 and 4. The eight susceptible isolates belonged to mixed clades such as 1 and 2, 1, 2 and 4 and 1 and 4. The resistant isolates were more homogeneous in terms of the clades to which they belonged. However, it cannot be suggested that a particular clade is driving resistance since the clades were distributed among susceptible and resistant isolates.

Conclusion: The current study provided information on the resistance patterns of clinical isolates of *G. vaginalis*. In this study a high level of resistance to metronidazole was observed. Approaches to approve the administration of clindamycin and vancomycin should be taken into consideration for the future treatment of BV in our current setting.

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SYMBOLS AND ABBREVIATIONS

%	Percentage
µL	Microliter
µM	Micromolar
˚C	Degrees Celsius
AMR	Antimicrobial resistance
Bp	Base pair
BREC	Biomedical Research Ethics Committee
BV	Bacterial vaginosis
Ct	Cycle threshold
DNA	Deoxyribonucleic Acid
MIC	Minimum Inhibitory Concentration
PCR	Polymerase Chain Reaction
pH	Power of Hydrogen
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
STI	Sexually Transmitted Infection
UDG	Uracil-DNA glycosylases

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CHAPTER 1
INTRODUCTION

A healthy vaginal microbiome is dominated by *Lactobacilli* species and has a low pH (Deng *et al*., 2018). When there is a fluctuation in pH, infections arise. Bacterial vaginosis (BV) has emerged as a global health threat that affects women of reproductive age. It causes abnormal vaginal discharge and is characterised by an unpleasant odour due to the decrease in the number of *Lactobacilli* accompanied by the overgrowth of anaerobic bacteria (Coudray & Madhivanan, 2020). BV has negative effects on the overall health of the individual. Proteolytic enzymes break down vaginal peptides, which increase vaginal transudation and damages epithelial cells, this causing vaginal discharge (Sobel, 2000). Women with BV may experience cervical inflammation and discomfort while other individuals are asymptomatic. Untreated cases of BV can lead to preterm delivery and increase the risk of contracting other sexually transmitted infections (STIs) such as Human immunodeficiency virus (HIV), herpes simplex type 2 infection, and gonorrhoea (Coudray & Madhivanan, 2020). Some characteristics of BV includes; a shift in fermentation products and metabolites, lower viscosity of vaginal fluid, detectable levels of the enzyme, *sialidase,* and the depletion of mucosal sialic acids (Morrill *et al*., 2020). Treatment of BV includes the use of broad-spectrum antibiotics such as metronidazole and clindamycin (Sousa *et al*., 2023).

Many studies have proven that one of the main microorganisms that plays a crucial role in BV is *Gardnerella vaginalis* (Pleckaityte *et al*., 2012). *G. vaginalis* is present in low numbers in the vaginal microbiome. Cells of *G. vaginalis* appear to be coccobacilli due to this bacterium being pleomorphic. The Gram-reaction is influenced via the physiological state of the bacteria resulting in Gram-positive to Gram-variable reactions. When grown *in vitro* on selective media plates, colonies appear small, grey, or opaque and are beta-haemolytic. *G. vaginalis* is a

facultative, anaerobic, non-motile and non-spore forming bacterium (Catlin, 1992). *G. vaginalis* has the ability to produce a biofilm that is responsible for recurrence of BV and contributes to antimicrobial resistance (Nagaraja, 2008).

G. vaginalis can be detected by molecular techniques such as the Polymerase Chain Reaction (PCR). The *16S ribosomal RNA* PCR and clade PCR assays have been used for the genotyping and identification of *G. vaginalis* via the amplification of specific genes. The clade PCR amplifies the *tuf* gene, which encodes for the translation elongation factor *Tu* (Balashov *et al*., 2014). Various TaqMan probes and qPCR primers were designed that target the *tuf* sequences conserved among *G. vaginalis* clades (Balashov *et al*., 2014). Currently, there are limited data on the clades of *G. vaginalis* isolates from our local setting. In this study, pure isolates of *G. vaginalis* obtained from pregnant women were genotyped and assigned to specific clades based on the sequence variations of their *tuf* gene. Women with BV have been suggested to have different clades of *G. vaginalis*, which influences their susceptibility or resistance to metronidazole (Jones, 2019). There is also a lack of data in our setting on antimicrobial resistance (AMR) patterns of isolates of *G. vaginalis*. In this study, AMR patterns to various antibiotics were determined, and the link between AMR patterns and the clades of *G. vaginalis* clades was also described in this study.

CHAPTER 2
LITERATURE REVIEW

2.1 Epidemiology of Bacterial Vaginosis and Impact on Health

2.1.1 Epidemiology

Bacterial vaginosis (BV) has emerged as a global health threat that affects women of reproductive age (Coudray & Madhivanan, 2020). Worldwide, there are almost one third of women who are positive for BV (Yalew *et al*., 2022). Studies have found that there is a higher prevalence of BV in developing countries such as Latin America, Asia, and most African countries, with the highest prevalence seen in African countries (Yalew *et al*., 2022).

According to Figure 1, the prevalence of BV in Europe and Central Asia was reported to be 22.8%, 24.2% for East Asia and the Pacific and 24.2% for Latin America and the Caribbean. For Sub-Saharan Africa, it was shown to be 24.6%. In the region of Sub-Saharan Africa, the highest prevalence of BV is in Southern and Eastern Africa (Kenyon *et al*., 2013). SubSaharan, Western and Central Africa had a lower prevalence (20. 6%) of BV compared to Southern and Eastern Africa (33.3%) (Peebles *et al*., 2019). Additionally, a study conducted in 2021, showed that the prevalence of BV ranged from 28.5% in Eastern Africa to 52.4% in Southern Africa (Nyemba *et al*., 2021).

Figure 1: Global prevalence of bacterial vaginosis (Peebles *et al*., 2019)

The prevalence of BV varies amongst different ethnic groups (Peebles *et al*. 2019) with prevalence estimates ranging from 10% to 50% depending on the population being studied (van de Wijgert *et al*., 2014). In a study conducted by Lewis (2011), it was reported that sexual behaviours such as multiple sex partners or douching have been associated with an increased risk of developing BV. Molecular studies have revealed that shifts in the microbial composition of the vaginal microbiota contribute to the development of BV (van de Wijgert *et al*., 2014). In South Africa, BV is considered a common disease, with an estimated prevalence ranging from 16% to 51% (Lewis, 2011). According to Mlisana *et al*. (2012), symptomatic vaginal discharge is a poor predictor of sexually transmitted infections and genital tract inflammation in highrisk women in South Africa. Studies suggest that the general prevalence of BV and its epidemiology in South Africa, as well as other countries, need more research in order to develop therapeutic vaccines and understand the risk factors, pathogenesis, and clinical implications of this polymicrobial syndrome (Bradshaw *et al*., 2016). According to Kenyon *et al*. (2013), the prevalence of BV varies by ethnic group within counties. In a study conducted in the United States, the prevalence of BV was highest in African Americans, lowest in

Caucasians and Asians, and intermediate within the Hispanic ethic group (Kenyon *et al*., 2013).

Additionally, a higher prevalence was observed for pregnant women compared to the general population with significant differences in the prevalence estimates for black and Hispanic women, as shown in Figure 2.

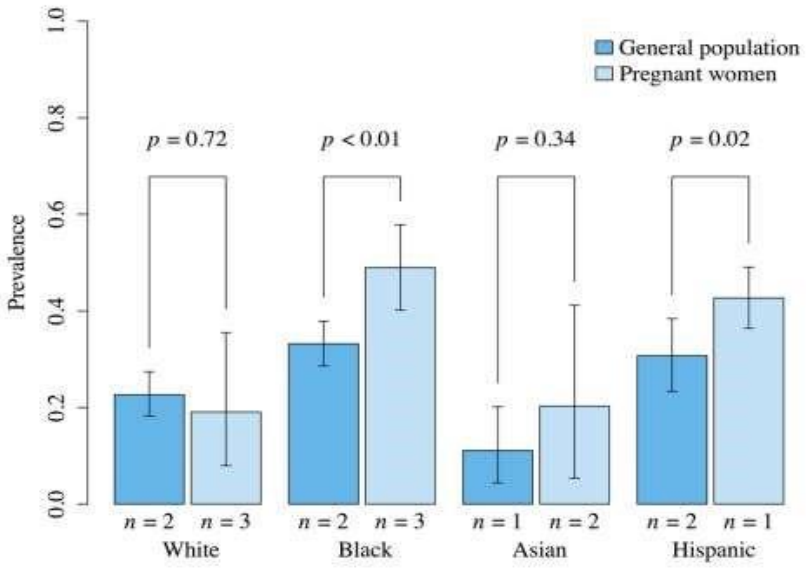


Figure 2: Prevalence of BV across the different ethnic groups in the United States (Peebles *et al*., 2019).

The risk factors for acquiring BV include vaginal douching, smoking, multiple sex partners, sexual intercourse before the age of 14, as well as a partner with BV (Shimaoka *et al*., 2019). Other risk factors associated with BV includes contraceptive use, antibiotic use, race, education, age and menstrual cycle (Coudray & Madhivanan, 2020). When BV is left untreated, it may lead to other complications such as premature delivery, intrauterine foetal death, pelvic inflammatory disease, and miscarriage (Shimaoka *et al*., 2019). When BV is untreated, it induces cervicitis or vaginitis, thus causing inflammation of the foetal membranes, leading to premature membrane rupture and labour (Shimaoka *et al*., 2019). Current studies suggest that BV is related to sexual activity (Coudray & Madhivanan, 2020). Factors such as; women who have sex with women, the use of sex toys, oral and anal sex, number of lifetime sexual partners,

frequency of vaginal intercourse, and history of bacterial STIs have increased the risk of acquiring BV (Coudray & Madhivanan, 2020).

2.1.2 Impact on health

BV causes vaginal discharge and is predominant in women of reproductive age (Tahir et al., 2022). It has many negative outcomes such as preterm birth, pelvic inflammatory diseases, and sexually transmitted diseases (Chen, Lu, Chen and Li, 2021). This condition has the ability to impact quality of life by causing symptoms such as vaginal itching, pain, odour, and burning (Bilardi *et al*., 2013). Immune responses to bacteria associated with BV ascending into the upper reproductive tract, as well as colonisation of the placenta, cause inflammation that has an effect on the health of new-borns (Onderdonk *et al*., 2016). The levels of pro-inflammatory cytokines are increased by bacteria associated with BV *in vitro*. This plays a role in enhancing the risk of HIV transmission by directly stimulating HIV replication in latent viral reservoirs (Onderdonk *et al*., 2016). BV has also been shown to have a negative effect on both mother and baby, as well as the overall reproductive health of the infected individual (Bhakta *et al*., 2021).

2.2 Diagnosis and Treatment of BV

2.2.1 Diagnosis

BV is clinically characterised by Amsel's criteria or by Nugent scoring (Amsel *et al*., 1983). The gold standard for the diagnosis of BV is the Nugent scoring method. Nugent scoring which assigns a score between 0 and 10 is based on the abundance of the following various bacterial morphotypes: large Gram-positive rods (*Lactobacillus* morphotypes), small Gramvariable rods (*G. vaginalis* morphotypes), small Gram-negative rods (Bacteroides *spp*. morphotypes), curved Gram-variable rods (*Mobiluncus* species morphotypes), and Grampositive cocci. Each morphotype is quantitated from 1 to 4+ based on the number of morphotypes per oil immersion field (0, no morphotypes; 1+, less than 1 morphotype; 2+, 1 to 4 morphotypes; 3+, 5 to 30

morphotypes; and 4+, 30 or more morphotypes). Scores between 0 and 3 represent "normal vaginal flora," scores between 4 and 6 represent "intermediate vaginal flora," and scores between 7 and 10 are considered positive for BV (Nugent *et al*., 1991).

Amsel's criteria involve saline microscopy. The diagnosis is based on the presence of a thin and homogenous watery discharge, elevated vaginal pH, the presence of more than 20% clue cells and a fishy odour after the addition of 10% potassium hydroxide (Amsel et al., 1983). A combination of these two techniques has the ability to ensure an accurate diagnosis of BV. According to Ezeigwe *et al*. (2023), there was a strong correlation between Nugent scoring and Amsel's (78.16% sensitivity, 92.68% specificity, 79.07% positive predictive value, 92.31% negative predictive value and 88.89% accuracy rate).

2.2.2 Treatment

Treatment for BV mainly makes involve the use of metronidazole, clindamycin or nitroimidazole. These are broad-spectrum antibiotics (Bradshaw & Sobel, 2016). These antimicrobials are administered orally or in cream form (Nagaraja, 2008). Studies have shown that the success rate with oral therapy of metronidazole and clindamycin has been 84-96%. The success rates for vaginal creams have been reported to be 75% and 86%, respectively (Nagaraja, 2008). Prebiotics are also used as an adjunct treatment for acute BV infection, which has been shown to deter recurrence (Jones, 2019). Regarding a more novel approach, thermoplastic polyurethane-based intravaginal rings are currently being investigated (Jones, 2019). It works on a similar concept to the NuvaRing and is responsible for delivering antimicrobials and lactic acid to the vaginal flora, thus preventing recurrent BV.

2.3 History of *G. vaginalis*

In 1953, Leopold discovered *G. vaginalis* and described it as a novel *Haemophilus*-like species that was associated with prostatitis and cervicitis (Catlin, 1992). A few years later, in 1955, Gardner and Dukes described that this specific microorganism is related to BV (Catlin, 1992).

The inability of this organism to grow on agar lacking blood had convinced researchers that they were dealing with a new *Haemophilus* bacterial species, thus it was named *Haemophilus vaginalis*.

The term "*vaginalis*" comes from its origin, the vagina (Turovskiy *et al*., 2011). Later, it was discovered that unlike other species belonging to the genus *Haemophilus*, *H. vaginalis* occasionally had a positive Gram reaction and did not require Nicotanimide adenine dinucleotide for growth (Turovskiy *et al*., 2011). This mysterious organism was then placed into the *Corynebacterium* genus and was referred to as *Corynebacterium vaginale* (Dunkelberg *et al*. 1970; Deane *et al*., 1972). It was later discovered that this specific bacterium did not conform to the *Corynebacterium* genus due to its catalase-negative reactions and the lack of arabinose in its cell wall (Catlin 1992). A new genus named *Gardnerella* was proposed by Greenwood and Pickett, *G. vaginalis* being the only bacterium in this genus (Catlin 1992).

2.4 Overview of G. vaginalis and its virulence factors

G. vaginalis has been reported to cause changes within the vaginal microbiome which influences the growth of other unwanted, anaerobic organisms, which together leads to the progression of infections such as BV (Morrill *et al*., 2020). *G. vaginalis* has various virulence factors which aid in the progression of infection and pathogenesis of BV. In addition to its role in BV, it causes other health conditions such as; urinary tract infections, pelvic inflammatory disease and preterm birth. According to Taylor & Phillips. (1983), beta-haemolysis of human blood provides a precise identification of *G. vaginalis*. It is beta-haemolytic in media containing human or rabbit blood, but not sheep blood (Catlin, 1992). *G. vaginalis* is a pleomorphic bacterium therefore the Gram-stain results vary from Gram-positive to Gram-variable. These pleomorphic rods are non-motile and do not produce endospores or capsules (Catlin, 1992). Both small cocci-bacilli and longer forms of cocci can be seen on 24hour cultures of *G. vaginalis* on blood agar plates (Catlin, 1992). *G. vaginalis* is a facultative anaerobic bacterium

and may have the ability to tolerate high oxidation-reduction potential present in a healthy vaginal microbiome (Schwebke *et al*., 2014). This bacterium begins the process of creating a lower redox potential, thus allowing for a suitable environment for the overgrowth of strict anaerobes that are present in low numbers in a healthy vaginal microbiome (Schwebke *et al*., 2014). It also requires specific nutrients to ensure growth. It has the ability to produce a biofilm that contributes to its overall ability to colonise the vaginal epithelium (Swidsinski *et al*., 2013). The genome of this bacterium is complex and there has been high genetic diversity between the different strains (van der Veer *et al*., 2019).

Many virulence factors have been identified in *G. vaginalis*. This includes; the production of vaginolysin, *sialidase,* and *bacteriocins*. *Sialidase* is responsible for breaking down the mucus layer of the vaginal epithelium (Ma *et al*., 2022). Other virulence factors of *G. vaginalis* includes; the ability to degrade and adhere mucin, surface hydrophobicity, microcapsule and *prolidase* activity (Pleckaityte *et al*., 2012). These virulence factors aid in outcompeting other microorganisms present in the vaginal microbiome (Patterson *et al*., 2007). The biofilm formation of this bacterium confers heightened antibiotic tolerance and resistance to host immune responses (Garcia *et al*., (2021). The initial steps in establishing infection within a host is adherence to the host receptor sites, biofilm formation, and the production of cytotoxic substances (Schwebke *et al*., 2014). In a study conducted by Garcia *et al*. (2021), it was shown that the vaginolysin of *G. vaginalis* is pro-inflammatory, thus contributing to the damage of the vaginal tissue in women with BV.

The production of a biofilm is essential for the survival of *G. vaginalis* in the vaginal microbiome. Studies have shown that the *sialidase* production enhances biofilm production via its *mucinase* activity (Schwebke *et al*., 2014). After a biofilm is established, the bacteria make use of quorum-sensing which aids in cell-to-cell communication (Schwebke *et al*., 2014). The presence of clue cells indicates the adhesion of the virulence factor, which aids in the

establishment of infection (Udayalaxmi *et al*., 2011). According to Udayalaxmi *et al*. (2011), the adherence and colonization of *G. vaginalis* are considered the initial stages in the pathogenesis of BV.

Studies have shown that *G. vaginalis* biofilms are more resistant to lactic acid and hydrogen peroxide compared to planktonic cells (Udayalaxmi *et al*., 2011). Therefore, biofilm formation influences colonisation as well as increases bacterial resistance to lactic acid and hydrogen peroxide. This is due to quorum sensing that allows bacteria to manage their community (Schwebke *et al*., 2014). Production of *phospholipase C* is another virulence factor, and it may allow damage of tissue from the reproductive tracts such as vaginal epithelial cells (Udayalaxmi *et al*., 2011). According to Udayalaxmi *et al*. (2011), the *phospholipase C*-mediated degradation of placental tissues triggers premature labour, which is a health concern associated with BV.

2.5 Transmission of *G. vaginalis*

The epidemiology profile of BV mimics that of an established STI (Verstraelen *et al*., 2010). *G. vaginalis* carriage is enhanced by both penetrative sexual contact as well as non-penetrative sexual contact and oral sex. Observations suggest that the carriage of *G. vaginalis* is due to female-to-male transmission rather than male-to-female transmission (Verstraelen *et al*., 2010). Verstraelen *et al*. (2010) suggested that BV can be considered as a sexually enhanced disease and that the frequency of intercourse is a critical factor in acquiring BV and the microorganisms associated with BV.

There have been various reports suggesting that BV as well as BV associated microbes are sexually transmitted. Figure 3 shows that *G. vaginalis* is not part of the acquired vaginal microflora at birth. However, transmission via sexual activities with an infected partner allows for the introduction of *G. vaginalis* into the vaginal microbiome (Schwebke *et al*., 2014). It is evident that sexual exposure plays a major role in the introduction of *G. vaginalis* to the vaginal

microbiome. Due to its virulence factors, *G. vaginalis* can adhere to the host's epithelium as well as establish a biofilm which aids in outcompeting *Lactobacilli*. According to Muzny *et al.* (2022), male circumcision reduces the risk of BV in women, as the penile microbiota is similar to that of the vaginal microbiota. Studies suggest that *G. vaginalis* colonises the male partners of women with BV (Schwebke *et al*., 2014). Females whose male partners have other partners were reported to have been associated with prevalent BV, supporting the statement that BV is sexually transmitted (Muzny *et al*., 2022). For women who have sex with women, it has been shown that both women have the same BV status, thus supporting the suggestion regarding sexual transmission of *G. vaginalis* (Muzny *et al*., 2019).

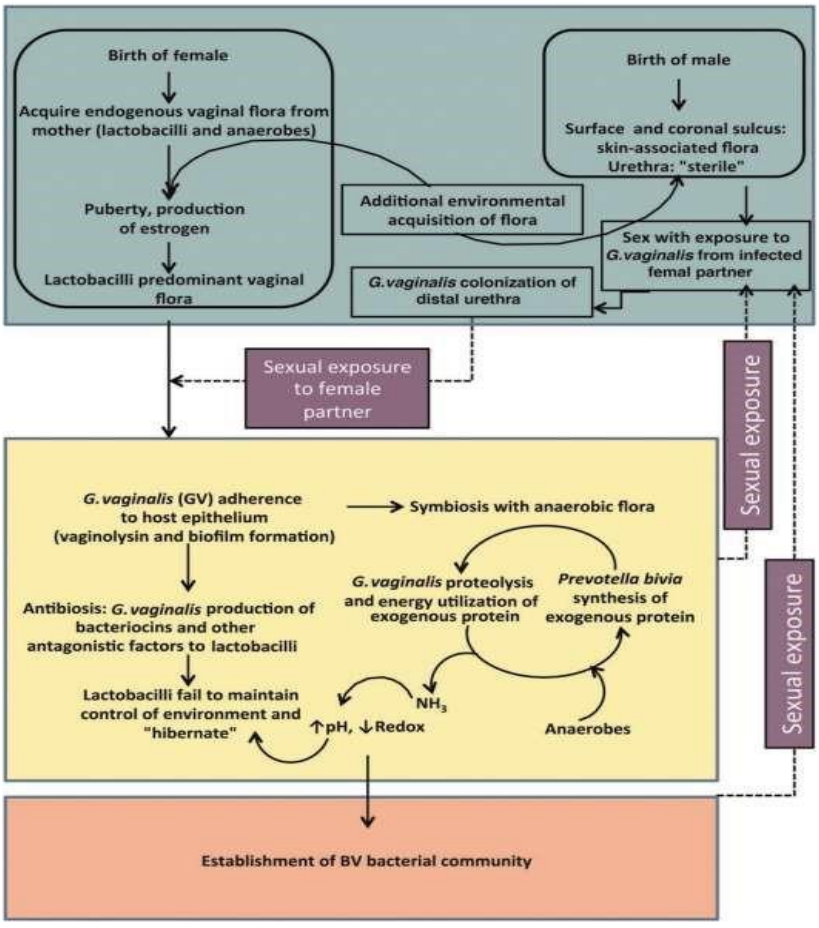


Figure 3: Establishment of the BV bacterial community showing how sexual exposure plays a role in acquiring *G. vaginalis* (Schwebke *et al*., 2014).

2.6 Antimicrobial Mechanism of action

Metronidazole works by diffusing into the microorganism and is responsible for inhibiting protein synthesis via the interaction with DNA (Weir & Le, 2022). This results in the loss of the double stranded, helical structure of DNA resulting in breakage and causing cell death (Figure 4). First, metronidazole enters the membrane of susceptible anaerobic cells. This is followed by step two, which involves reductive activation by intracellular transport proteins which is achieved by altering the chemical structure of *pyruvate-ferredoxin oxidoreductase* (Weir & Le, 2022). A concentration gradient is established when metronidazole undergoes reduction, causing the cells to take up more drugs, which promotes free radical formation (Weir & Le, 2022). The third step involves the interactions with intracellular targets, which are achieved by cytotoxic particles reacting with the host cell DNA. This results in the breakage of the DNA strand as well as the double helix. The fourth step involves the breakdown of cytotoxic products (Figure 4) (Weir & Le, 2022).

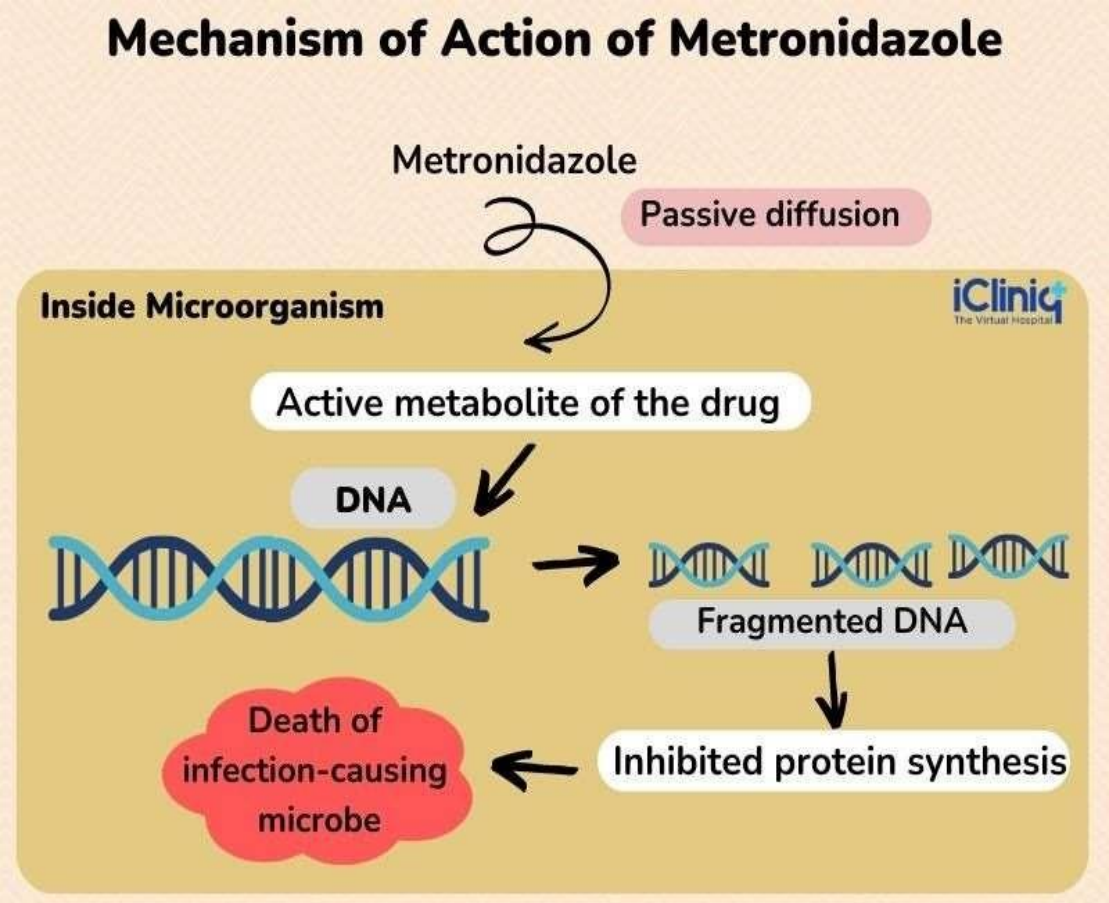


Figure 4: Mechanism of metronidazole on bacterial cells (Bhavsar, 2022).

The mechanism clindamycin uses to be bactericidal or bacteriostatic is preventing peptide bond formation (Murphy *et al*., 2022). These mechanisms depend on the organism, infection site, and drug concentration. With regards to *G. vaginalis*, clindamycin has a bacteriostatic effect. Clindamycin does not spontaneously act as bacteriostatic or bactericidal. The activity of clindamycin is dependent on the organism, infection site, and drug concentration. With regards to *G. vaginalis*, clindamycin has a bacteriostatic effect as it is a bacterial protein synthesis inhibitor. This results in inhibition of protein synthesis via the reverse binding to the *50S ribosomal* subunits, as seen in Figure 5 (Bunyan *et al*., 2019). The absorption of clindamycin administered orally cannot occur until it is hydrolysed within the gastrointestinal tract. After absorption, it is distributed throughout the body. In the bloodstream, it is bound to proteins and is primarily metabolised by CYP 3A4 and CYP 3A5 present in the liver. These compounds aid

in the oxidation of clindamycin to clindamycin sulfoxide and N-dimethyl clindamycin (Bunyan *et al*., 2019). When looking at clindamycin that is administered orally, an oxidation of clindamycin to clindamycin sulfoxide and N-dimethyl clindamycin occurs in the liver. This is due to the fact that clindamycin cannot be absorbed in its primary form. Therefore, in order for clindamycin to be effective and absorbed by the body, it is hydrolysed into compounds that have the same effects on bacterial cells (bacteriostatic).

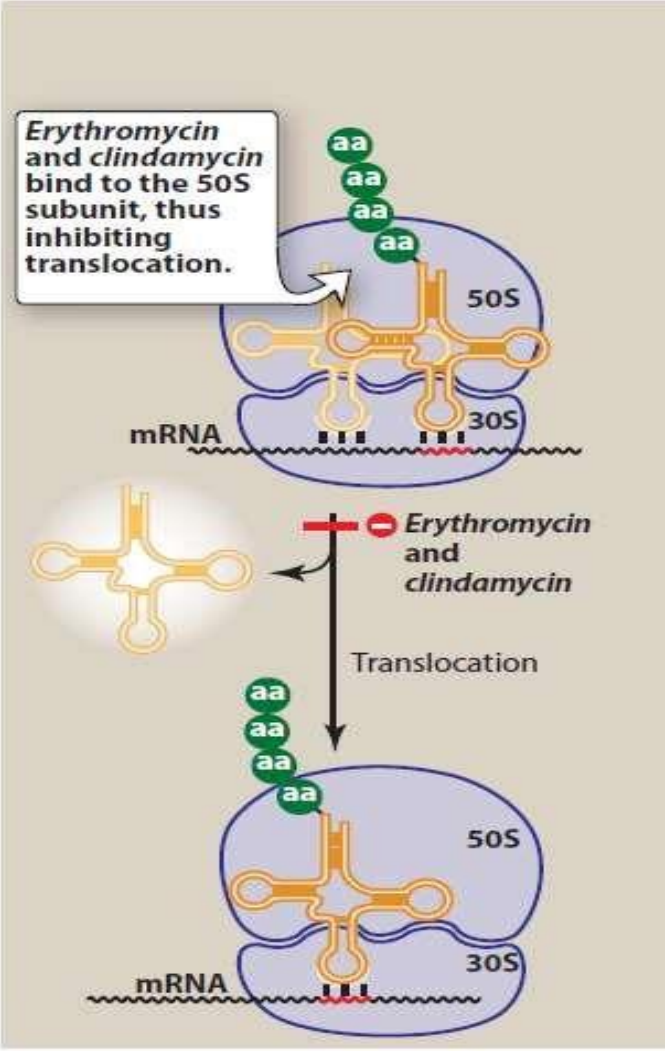


Figure 5: Mechanisms of clindamycin in bacterial cells (Rima, 2022).

Vancomycin exerts bactericidal effects via the inhibition of the polymerisation of peptidoglycan present in the bacterial cell wall (Patel *et al*., 2022). Bacterial cell walls are composed of a peptidoglycan layer and polymers of N-acetylglucosamine (NAG) and

Nacetylmuramic acid (NAM),respectively (Figure 6). This antimicrobial binds to D-alanine, resulting in the inhibition of peptidoglycan synthase and P-phospholipid carrier (Patel *et al*., 2022). This has adverse effects, as it prevents the synthesis as well as polymerization of NAM and NAG. This results in a weakened bacterial cell wall which influences the leakage of intracellular molecules, causing bacterial cell death (Patel *et al*., 2022).

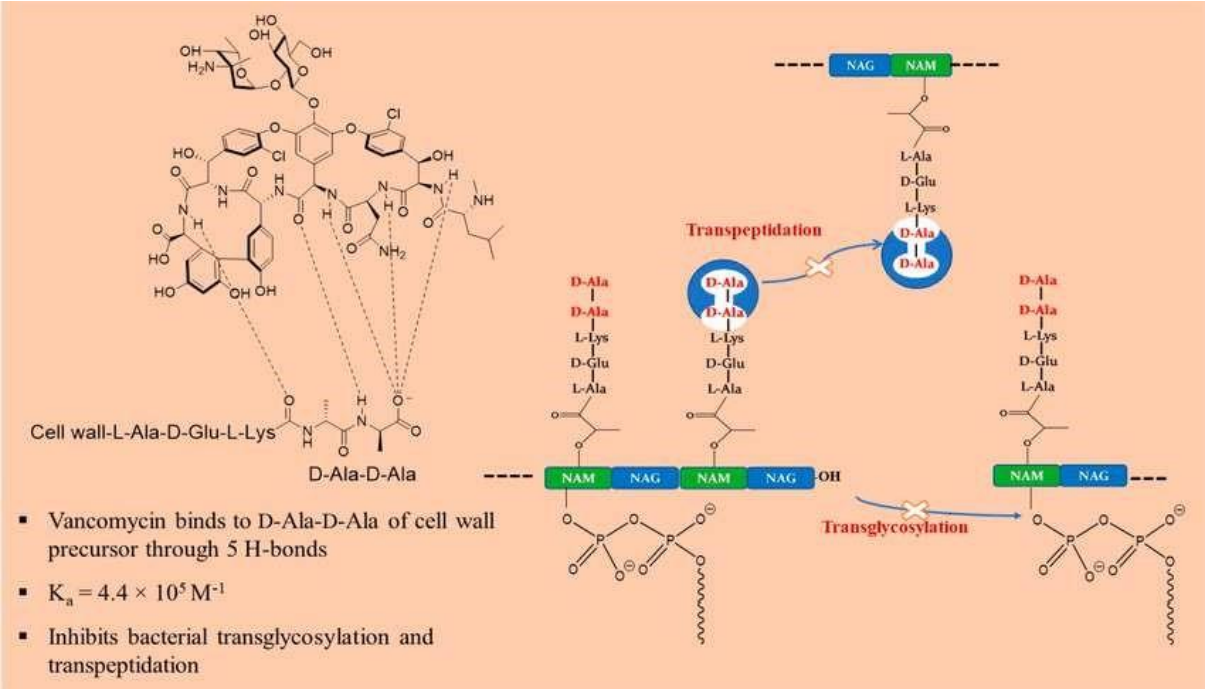


Figure 6: Mechanisms of vancomycin on bacterial cells (Dhanda *et al*., 2018).

2.7 Resistance Patterns

Metronidazole, clindamycin, and vancomycin have been used for the treatment of BV. However, new forms of treatment are required due to the antimicrobial resistance of *G. vaginalis*. With repeated administration of the antimicrobials, there is an increase in antibiotic resistance (Nagaraja, 2008).

There are many studies that aid in understanding *G. vaginalis* resistance to metronidazole. In a study conducted by Schuyler *et al*. (2016), four *G. vaginalis* clades have been identified. Previous research has suggested that two out of the four *G. vaginalis* clades are resistant to metronidazole (Jones, 2019). Women with BV have been suggested to harbour different clades

of *G. vaginalis*, which influences their susceptibility or resistance to metronidazole (Jones, 2019). In a study conducted by Nagaraja (2008), a 68% resistance of *G. vaginalis* to metronidazole was observed, as well as 30% recurrent BV. Recurrence is due to the survival of metronidazole-resistant bacteria present in the vagina after treatment. In a study conducted by Ma *et al*. (2022), samples collected in Northeast China showed a higher resistance to metronidazole than to clindamycin. The resistance rates to metronidazole reached 87.5% and the resistance rates to clindamycin reached 33.3%. Regarding recurrent BV, in a study conducted by Nagaraja (2008), results concluded that among the strains isolated from recurrent BV, 58.8% were resistant to metronidazole. A study by Simoes *et al*. (2001) observed different biotypes and genotypes of *G vaginalis* among clinical isolates. The study findings showed a 64% resistance of *G. vaginalis* to metronidazole.

Researchers have hypothesized many reasons as to how and why *G. vaginalis* acquires resistance to metronidazole. One hypothesis considers the treatment of patients using metronidazole, allowing for the selection of resistant strains (Simoes *et al*., 2001). Another study showed that *G. vaginalis* became resistant to metronidazole when grown in its presence for more than five subcultures, indicating that the required genes for resistance have been acquired (Simoes *et al*., 2001). Concern of metronidazole resistance has been increasing due to the widespread use of this antimicrobial for the treatment of BV. Some reports have shown that there are certain strains of *G. vaginalis* that show resistance to clindamycin.

However, resistance rates to metronidazole are much higher than those of clindamycin.

There is no evidence suggesting that *G. vaginalis* is resistant to vancomycin. To date, clindamycin and vancomycin are being used to treat BV and recurrent BV.

2.8 Genes associated with resistance

Studies have found that antimicrobial resistant genes (AMR) were more common in the vaginal microbiome of women with BV rather than symptomatic women without BV (Bostwick *et al*.,

2016). There are four major classes of *erm* genes (*ermA, ermB, ermC, and ermF*). However, the *ermA* gene is the primary gene responsible for antimicrobial resistance associated with clindamycin in *G. vaginalis*. In a study conducted by Bostwick *et al*. (2016), the gene *ermTR* is responsible for resistance to lincomycins and the genes *nim* and *nimB* are responsible for resistance to nitroimidazoles. The genes *ermTR* and *tet* comprise the majority of antimicrobial resistance genes (Bostwick *et al*., 2016). According to Beigi *et al*. (2004), *nim* genes are associated with the resistance of *G. vaginalis* to metronidazole.

In a study conducted by Schellenberg *et al*. (2016), the *cpn60* gene was used to identify subgroups of *G. vaginalis* with distinct genetic profiles and *sialidase* activity. This could have implications for antibiotic resistance and disease pathogenesis. Several genes have been identified to play a role in antibiotic resistance in *G. vaginalis*. The genes encode enzymes or transporters that modify or remove antibiotics, leading to reduced susceptibility. Genes associated with antibiotic resistance are *tet(M), tet(O), erm(B), and Inu(A).*

Nim genes encode a *reductase* that converts nitroimidazole into a non-toxic derivative. This type of gene is usually found on plasmids (Bostwick *et al*., 2016). However, recent studies have shown that *nim* genes are also found on bacterial chromosomes and can be passed to the next bacterium via a conjugative process (Bostwick *et al*., 2016). *Erm* genes are responsible for modifying the structure of the antibiotic-binding site (Bostwick *et al*. 2016) and are responsible for inhibiting bacterial protein synthesis. The *TetM* determinant encodes a high-level resistance to tetracycline, and this gene is located on the chromosome (Huang *et al*., 1997). Studies show that this gene is also associated with conjugative transposons and has the ability to be plasmid encoded. Regarding *G. vaginalis*, resistance to high-level tetracycline, is carried on the chromosome (Huang *et al*., 1997). According to a study by Huang *et al*. (1997), four tetracycline-resistant strains possessed *tetM* genes and had identical nucleotide sequences. The study also concluded that the nucleotide sequences of the two versions of the *tetM* gene present

in *G. vaginalis* are not 100% identical to the published sequences. Therefore, it suggests that the differences in the *tetM* gene in *G. vaginalis* share the identity with segments of the gene from different sources. In a study by Zhang *et al*. (2022), all *Gardnerella* isolates contained antibiotic resistant genes. Some isolates contained the macrolide erythromycin resistance gene *ermX*, others contained the lincosamide resistant gene *IsaC* and other isolates contained the tetracycline resistant gene *tetL* and *tetM* (Zhang *et al*., 2022).

2.9 Rationale

Bacterial vaginosis (BV) is gaining interest due to its problematic outcomes in symptomatic women of childbearing age. The most common symptom is abnormal grey discharge that has a fishy odour accompanied by itchiness and pain. BV is a result of a decrease in the abundance of hydrogen peroxide producing *Lactobacilli* in the vaginal microbiome. *Lactobacilli* ensure the protection of the vaginal microbiome by decreasing infections. *G. vaginalis* is said to be the main species of bacteria involved in BV and is responsible for its pathogenicity. *G. vaginalis* has many virulence factors. Currently, there are limited data on the AMR patterns of isolates of *G. vaginalis* in our local setting. In this study, AMR patterns to various antibiotics will be determined as well as genetic typing of the isolates. In this study, the link between AMR patterns and *G. vaginalis* genotypes (clades) was be described.

2.10 Aim

To investigate the antimicrobial susceptibility patterns and genotypes (clades) of clinical isolates of *G. vaginalis*.

2.11 Objectives

1) To determine the antimicrobial patterns of isolates of *G. vaginalis* to various antibiotics (Penicillin, amoxicillin, clavulanic, piperacillin, tazobactam constant, cefoxitin, imipenem, chloramphenicol, erythromycin, clindamycin, metronidazole, moxifloxacin, tetracycline, and vancomycin).

2) To determine the genotypes of the isolates based on sequence variation of the *tuf* gene.

3) To link antimicrobial patterns to BV status and genotypes (*G. vaginalis* clades).

CHAPTER 3
METHODOLOGY

3.1 Ethical Statement

Ethical approval for this study was obtained from the Biomedical Research Ethics Committee (BREC), University of KwaZulu-Natal (BREC/00005700/2023).

3.2 Study design and sample size

This was an experimental laboratory-based study. Pregnant women who attended care at the King Edward VIII Hospital in Durban, South Africa were included in this study. The samples were collected from January to August 2022. To participate in the study, the women had to be 18 years of age or older. For this study, a total of 150 women were enrolled (n=150). The women provided their written consent, as well as two self-collected vaginal swabs as instructed by the study team. Data on participants sexual behaviour, sociodemographic information, and clinical history were obtained from all enrolled women from gestational ages
12 weeks to 37 weeks. Following sample collection, the first swab was placed in Amie's transport medium (Copan, Brescia, Italy) and the second swab was rolled onto a glass slide. This glass slide was used to determine the BV state using Nugent scoring on Gram-stained vaginal smears.

3.3 Laboratory Testing

3.3.1 Diagnosis of BV status

The method used for the diagnosis of BV was Nugent scoring. The primary swab was dragged across the microscope slide and Gram-stained. The slides were heat-fixed to ensure that the bacteria adhered to the surface of the slide. The slides were then stained with the primary stain,

crystal violet for 1 minute. After 1 minute, the crystal violet was washed with deionised water. Grams iodine was added to the slides. After 1 minute, the iodine was washed with acetone. The acetone was immediately rinsed off the slides with deionized water and the counterstain, carbol fuchsin, was added to slides for 1 minute. After 1 minute, the carbol fuchsin was rinsed with deionized water and the slides were gently dabbed with paper towel to remove excess water. The slides were viewed under a microscope where the peptidoglycan layers were observed. Gram-positive bacteria contain a thicker peptidoglycan layer and retain crystal violet, Gram-negative bacteria contain a thinner peptidoglycan later and cannot retain primary stain, therefore, stained pink due to counterstain carbol fuchsin.

The Nugent scoring worked on a scale of 0 to 10 and comprised of three components. A score of 0-4 indicates the presence of rod-shaped Gram-positive *lactobacilli*. A score of 4-6 indicating a greater number of gram-negative and Gram-variable bacteria, and a score of 7-10, which reflects the presence of curved rods. The scoring was based on the presence of different bacterial morphotypes. A score ≥7 indicated the presence of BV, scores from 4-6 indicated intermediate BV and 0-3 indicated normal or BV negative.

3.3.2 Bacterial Isolates

After diagnosis, the second vaginal swab was streaked on *G. vaginalis* selective agar as well as sheep blood agar plates. The *G. vaginalis* selective medium plates contained a blood agar base, Tween 80, gentamicin sulphate, nalidixic acid, amphotericin B, and human erythrocytes. The sheep blood plates contained agar, casein peptone, sodium chloride, soy peptone, sheep blood, and demineralised water. The plates were incubated for 48 hours at 36˚C with a CO_2 gas pack, in an anaerobic jar. After 48 hours, the plates were analysed for beta-haemolysis. If haemolysis was present on the sheep blood plate; the colony was discarded as *G. vaginalis* is non-haemolytic on sheep blood agar. Only colonies showing beta-haemolysis on the selective agar

plates were further analysed. The suspected *G. vaginalis* colonies were Gram-stained. After staining, the *G. vaginalis* colonies were picked up and sub-cultured on *G. vaginalis* selective agar plates under aseptic conditions. The plates were incubated anaerobically for 48-72 hours at 36˚C with a CO_2 gas pack.

3.3.3 Confirmatory Tests

Confirmatory assays included; Gram staining, amplification of the *16S rRNA* of *G. vaginalis*, catalase and oxidase tests. The Gram-staining procedure has been described in Section 3.3.1. The catalase test was performed using hydrogen peroxide. The colonies were removed from the culture plate with a sterile bamboo stick and placed in a test tube containing 3% hydrogen peroxide. The oxidase test involved the use of oxidase strips. Culture was picked from plates with a bamboo stick and placed onto the strip. The strip was impregnated with N, Ndimethyl-phenlenediamine and alpha-nephthol. Positive reactions were indicative of a colour change from colourless to purple. After the confirmatory tests were completed, the pure isolates were used for DNA isolation or scrapped from the plate and resuspended in a cryovial containing Brain Heart Infusion (BHI) broth for storage. The cultures were stored at -80˚C until further use.

3.3.4 DNA isolation

DNA was extracted using the Quick-DNA™ Miniprep Kit (Zymo Research, Catalogue nos. D3024 & D3025). DNA was extracted at room temperature and all centrifugation steps were performed at 14000 x g. *G. vaginalis* cultures were scraped from each plate and resuspended in a tube containing 2 ml brain-heart-infusion (BHI) broth that was acclimatised to room temperature. The samples were centrifuged for 10 minutes using an Eppendorf microfuge. After 10 minutes, the supernatant was discarded. Beta-mercaptoethanol was added to the

genomic lysis Buffer to a final dilution of 0.5 % (v/v), i.e., 250 µl per 50 ml. A volume of 500 µl of genomic lysis buffer was added to each sample. The samples were vortexed for 6 seconds and then left to stand at room temperature for 10 minutes. The mixture was transferred to a Zymo-Spin™ Column placed in a collection tube. The samples were then centrifuged for 1 minute. The collection tube was discarded with the flow through. The Zymo-Spin™ Column was transferred to a new collection tube. A volume of 200 µl of DNA pre-wash buffer was added to the spin column and was centrifuged for 1 minute. Thereafter, 500 µl of gDNA Wash Buffer was added to the spin column and was centrifuged for 1 minute. The spin column was transferred to a clean microcentrifuge tube. Thereafter, 50 µl of DNA elution buffer was added to the spin column and incubated for 5 minutes at room temperature, then centrifuged at top speed for 1 minute to elute the DNA. The concentration of the extracted DNA was determined using the Nano drop Spectrophotometer (ThermoFisher Scientific). For quantification, 2 µl of the elution buffer was used as a blank, thereafter 2 µl of the extracted DNA of each isolate was analysed and the results were recorded. Genomic DNA was stored at -20˚C.

The *16S rRNA* gene from *G. vaginalis* was used as a confirmatory test using specific primers described by Fredricks *et al*. (2007). The primer sequences that were used in the real-time PCR assay are shown in Table 1. Each PCR reaction was performed to a final volume of 10 µl. The composition of each reaction was 5 µl of PowerUp SYBR green mix (ThermoFisher Scientific, United States), 10 µM of the forward and reverse primers, 1:10 diluted template DNA, and 4 µl of nuclease-free water. PCR amplification was performed on the Quant Studio 5 Real-Time PCR detection system (ThermoFisher Scientific, United States). Reactions were performed on a 96-well plate. The cycling conditions consisted of Uracil-DNA glycosylases activation at 50˚C for 2 minutes, initial denaturation at 95˚C for 2 minutes, denaturation at 95˚C for 15 seconds, annealing at 55˚C for 15 seconds and extension at 72˚C for 1 minute. The PCR was performed with a total of 35 cycles. A negative control was added to assess the purity of

the PCR. PowerUp is a universal master mix and is used for real- time PCR. The master mix consists of Taq DNA polymerase, heat-labile uracil DNA glycosylase (UDG), dUTP/dTTP and ROX™ passive reference.

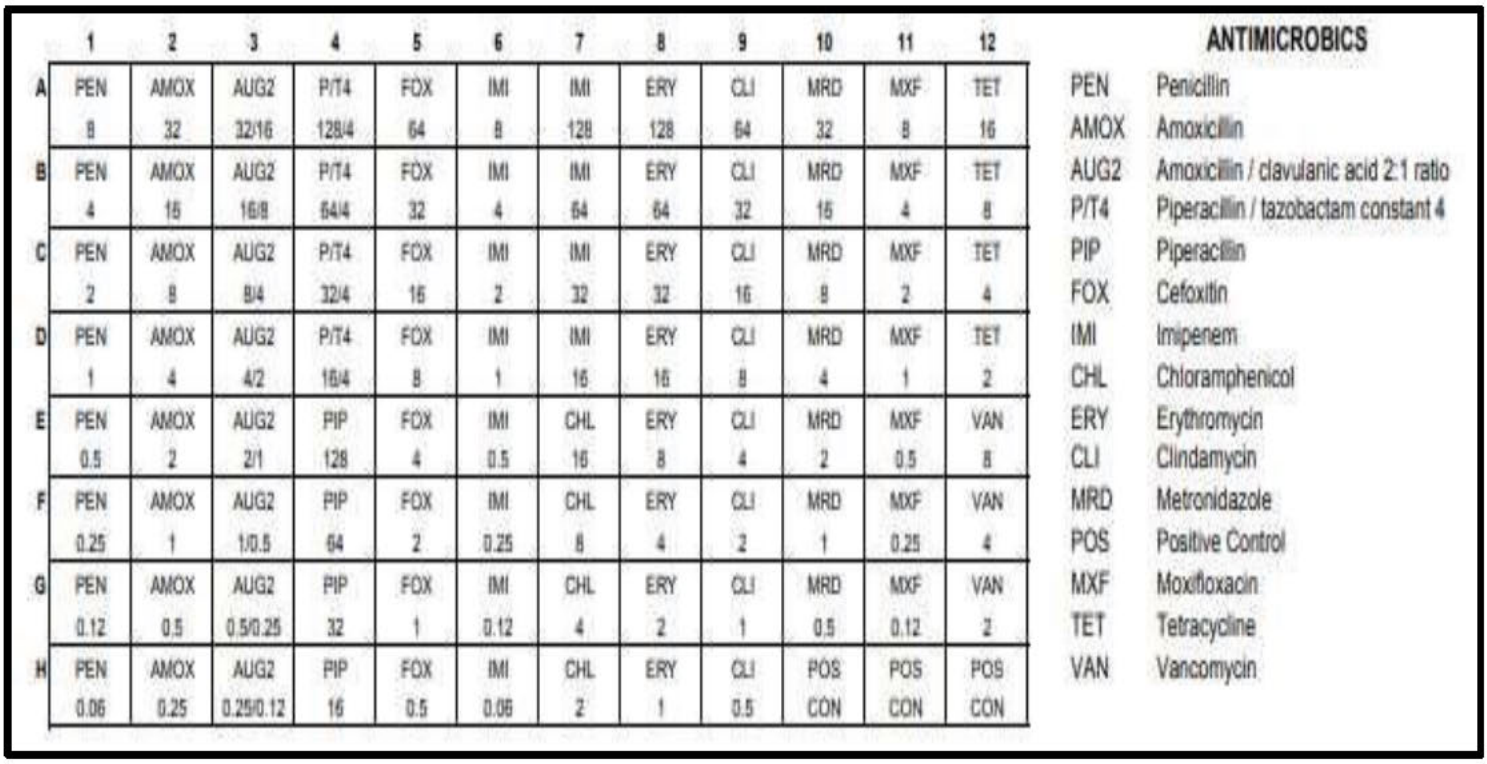
Table 1: Sequences of the *16S rRNA* primers used in this study.

Primer Name	Sequence (5'-3')
G. vag644F	GGGCGGGCTAGAGTGCA
G. vag851R	GAACCCGTGGAATGGGCC

3.3.5 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the Sensititre plate method (ThermoFisher Scientific). The pure isolates were grown from storage on chocolate agar plates that were composed of a GC agar base with haemoglobin and KoEnzyme enrichment. The plates were incubated anaerobically with the lid facing upwards, for 72 hours at 36˚C with a CO_2 gas pack. After the incubation period, 3-5 colonies were picked from the agar plate and emulsified in 5 ml of Thermo Scientific™ Mueller Hinton broth. The suspension was adjusted to 0.5

McFarland equivalent with the aid of the Thermo Scientific™ Sensititre™ Nephelometer. A volume of 100 µl was transferred to 11 ml Thermo Scientific™ Supplemented *Brucella* Broth for anaerobes. The 96-well plate contained various antibiotics in each well (Figure 7). The panel with 100 µl per well was incubated using the Thermo Scientific ™ Sensititre AIM™ Automated Inoculation Delivery System. All wells were covered with a perforated adhesive seal and incubated in an anaerobic jar for 46-48 hours at 34-36˚C. After the incubation period, the growth was analysed with the aid of a MIC mirror and the results were recorded accordingly.



	1	2	3	4	5	6	7	8	9	10	11	12
A	PEN 8	AMOX 32	AUG2 32/16	P/T4 128/4	FOX 64	IMI 8	IMI 128	ERY 128	CLI 64	MRD 32	MXF 8	TET 16
B	PEN 4	AMOX 16	AUG2 16/8	P/T4 64/4	FOX 32	IMI 4	IMI 64	ERY 64	CLI 32	MRD 16	MXF 4	TET 8
C	PEN 2	AMOX 8	AUG2 8/4	P/T4 32/4	FOX 16	IMI 2	IMI 32	ERY 32	CLI 16	MRD 8	MXF 2	TET 4
D	PEN 1	AMOX 4	AUG2 4/2	P/T4 16/4	FOX 8	IMI 1	IMI 16	ERY 16	CLI 8	MRD 4	MXF 1	TET 2
E	PEN 0.5	AMOX 2	AUG2 2/1	PIP 128	FOX 4	IMI 0.5	CHL 16	ERY 8	CLI 4	MRD 2	MXF 0.5	VAN 8
F	PEN 0.25	AMOX 1	AUG2 1/0.5	PIP 64	FOX 2	IMI 0.25	CHL 8	ERY 4	CLI 2	MRD 1	MXF 0.25	VAN 4
G	PEN 0.12	AMOX 0.5	AUG2 0.5/0.25	PIP 32	FOX 1	IMI 0.12	CHL 4	ERY 2	CLI 1	MRD 0.5	MXF 0.12	VAN 2
H	PEN 0.06	AMOX 0.25	AUG2 0.25/0.12	PIP 16	FOX 0.5	IMI 0.06	CHL 2	ERY 1	CLI 0.5	POS CON	POS CON	POS CON

ANTIMICROBICS

PEN	Penicillin
AMOX	Amoxicillin
AUG2	Amoxicillin / clavulanic acid 2:1 ratio
P/T4	Piperacillin / tazobactam constant 4
PIP	Piperacillin
FOX	Cefoxitin
IMI	Imipenem
CHL	Chloramphenicol
ERY	Erythromycin
CLI	Clindamycin
MRD	Metronidazole
POS	Positive Control
MXF	Moxifloxacin
TET	Tetracycline
VAN	Vancomycin

Figure 7: The Anaerobic reference card for the 96-well sensititre plate used to access antimicrobial susceptibility (ANAERO3-Thermo Fisher Scientific).

3.3.6 Genotyping Assays

The *tuf* gene associated with the coding elongation factor *Tu* in *G. vaginalis* was amplified. This gene aids in protein synthesis in bacteria. In this study, primer sets targeting four clades of *G. vaginalis* described by Bunyan *et al.* (2019) were used. The primer sets are shown in Table 2. This reaction was performed on the isolates, using a 96-well plate. Due to the small sample size, all four clades were analysed on a 96-well plate (Wells A1-G7). Each PCR reaction was performed in a final volume of 10 µl. It consisted of 5µl PowerUp, 0.25µl of each forward and reverse primer, 2.5 µl of nuclease-free water and 2 µl of diluted template DNA 1:10. A volume of 8 µl of the master mix was pipetted into each sample well and 10 µl of the master mix was pipetted into the negative control wells (A1, C1, E1, G1) for each clade. A volume of

2 µl of diluted DNA was added to each sample well (A3- B6, C3-D6, E3-F6, G3- H6). PCR amplification was performed on the Quant Studio 5 Real-Time PCR detection system. The cycling conditions were as follows; UDG activation at 50˚C for 2 minutes, initial denaturation at 95˚C for 2 minutes, denaturation at 95˚C for 15 seconds, annealing at 55˚C for 15 seconds, and the final step was extension at 72˚C for 1 minute. The total number of cycles performed was 35. A negative control was added to assess any contamination and integrity of the PCR.

Table 2: Sets of primers used in this study for amplification of the *tuf* gene for the assignment of the different clades of *G. vaginalis*

Primer Name	**Sequence (5'-3')**
GV Clade1F	CCAGTCATAAGTTTGCGTTTTACC
GV Clade1R	TGGCACTGGCAAAGTTTACAAC
GV Clade2F	GCAAAGCAGACTGAGCGTATTAG
GV Clade2R	GTAATAATCAGGCTCCTCATCGC
GV Clade3F	TTCTGCTTCTTCTGCTATTTGCTG
GV Clade3R	TTCGTTGACTTTTGGGCAACATG
GV Clade4F	CCTACGCAAGCTCCAGACGAC
GV Clade4R	ACAAGTTGCACTCTTCGAGCTGG

CHAPTER 4
RESULTS

4.1 Diagnosis of BV

The 150 vaginal swabs were screened for the diagnosis of BV using Nugent scoring. Figure 8 represents an image of the gram-stained slide. The presence of clue cells is shown in Figure 8. Of the 150 women, 49.3% (74/150) of the women were diagnosed as BV negative, 22.0% (33/150) were BV positive, and 28.7% (43/150) were BV intermediate.

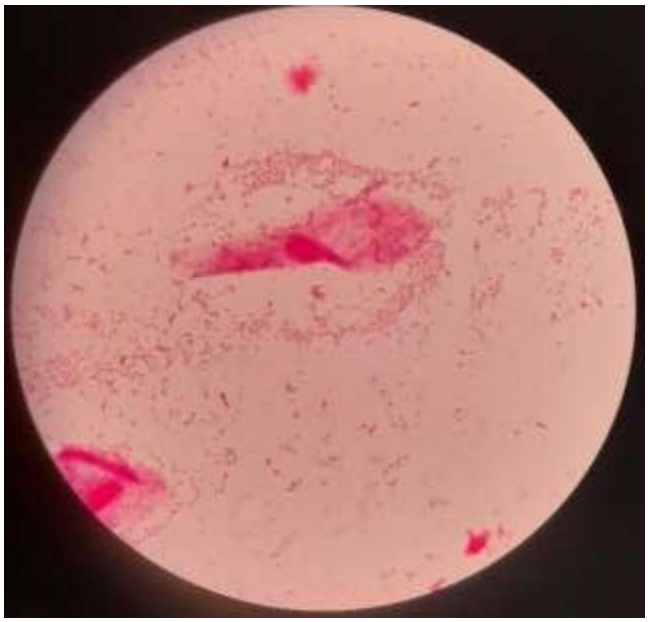


Figure 8: Primary Gram-stain showing vaginal epithelial cells *with G. vaginalis* attached to the cells (clue cells).

4.2 Factors associated with BV

Table 3 describes the characteristics of the study population according to BV status. There was a significant association between BV status and having symptoms of abnormal vaginal

discharge, p=0.025. Of the women who were BV negative, the majority 75.7% did not report having any discharge symptoms compared to 24.3% of the negative women who reported having symptoms. Of the women who were BV positive, the majority (84.8%) were asymptomatic (no discharge symptoms) when compared to 15.2% who had discharge. Similarly, for the BV intermediate women, a higher percentage (58.1%) were asymptomatic compared to 41.9% who had discharge (Table 3).

There was a borderline significance between condom use at the last sex act and BV status, p=0.057. The majority (84.8%) of the BV positive women reported not using a condom at last sex act compared to 15.2% who reported using condoms. Similarly, for the BV intermediate women, the majority (72.1%) reported not using a condom at last sex act compared to 27.9% who reported using condoms. For women diagnosed as negative for BV, 62.2% reported not using condoms compared to 37.8% who used condoms at the last sex act (Table 3).

All remaining variables were not significantly associated with BV status, p>0.05. Despite not being significant, the majority of the women across all BV states had attended high school, were unmarried, reported having a regular sex partner, did not cohabit with their partner, had experienced first sex between the ages of 15-20 years, had between 2-4 lifetime sex partners, were unaware if their partner had other partners and reported only sometimes using condoms. Furthermore, in all BV states, most women were not engaged in intravaginal practices, were in their third trimester of pregnancy, had no previous history of negative pregnancy outcomes and were not treated for STIs in the past. Compared to BV positive and negative women, a higher percentage of BV intermediate women reported having abnormal vaginal discharge in the past (Table 3).

Table 3: Characteristics of the study population stratified by BV status

Variables	BV Negative	BV Positive	BV Intermediate	Total	
	N (%)	N (%)	N (%)	N (%)	p-value
Overall	74 (49.3)	33 (22.0)	43 (28.7)	150 (100.0)	
Current abnormal discharge					0.025
No	56 (75.7)	28 (84.8)	25 (58.1)	109 (73.0)	
Yes	18 (24.3)	5 (15.2)	18 (41.9)	41 (27.0)	
Highest level of education					0.768
College	19 (25.7)	5 (15.2)	11 (25.6)	35 (23.3)	
High School	52 (70.3)	27 (81.8)	30 (69.8)	109 (72.7)	
Primary	3 (4.1)	1 (3.0)	2 (4.7)	6 (4.0)	
Marital status					0.599
No	67 (90.5)	31 (93.9)	41 (95.3)	139 (92.7)	
Yes	7 (9.5)	2 (6.1)	2 (4.7)	11 (7.3)	
Has a regular sex partner					0.783
No	14 (18.9)	6 (18.2)	6 (14.0)	26 (17.3)	
Yes	60 (81.1)	27 (81.8)	37 (86.0)	124 (82.7)	
Living with partner					0.954
No	50 (67.6)	22 (66.7)	30 (69.8)	102 (68.0)	
Yes	24 (32.4)	11 (33.3)	13 (30.2)	48 (32.0)	
Age of first sex					0.903
15-20	52 (70.3)	26 (78.8)	32 (74.4)	110 (73.3)	
21-25	19 (25.7)	5 (15.2)	9 (20.9)	33 (22.0)	
<15	2 (2.7)	1 (3.0)	1 (2.3)	4 (2.7)	
>25	1 (1.4)	1 (3.0)	1 (2.3)	3 (2.0)	
Life-time number of sex partners					0.067
1	12 (16.2)	3 (9.1)	11 (25.6)	26 (17.3)	

2 to 4	52 (70.3)	22 (66.7)	20 (46.5)	94 (62.7)	
>4	10 (13.5)	8 (24.2)	12 (27.9)	30 (20.0)	
Partner has other partners					0.550
No	32 (43.2)	10 (30.3)	14 (32.6)	56 (37.3)	
Yes	12 (16.2)	7 (21.2)	6 (14.0)	25 (16.7)	
Don't know	30 (40.5)	16 (48.5)	23 (53.5)	69 (46.0)	
Condom use					0.508
Never/rarely	27 (36.5)	9 (27.3)	12 (27.9)	48 (32.0)	
Sometimes	47 (63.5)	24 (72.7)	31 (72.1)	102 (68.0)	
Condom use at last sex act					0.057
No	46 (62.2)	28 (84.8)	31 (72.1)	105 (70.0)	
Yes	28 (37.8)	5 (15.2)	12 (27.9)	45 (30.0)	
Circumcised partner					0.835
No	69 (93.2)	30 (90.9)	41 (95.3)	140 (93.3)	
Yes	5 (6.8)	3 (9.1)	2 (4.7)	10 (6.7)	
Intravaginal practices					0.297
No	**62 (83.8)**	**25 (75.8)**	**31 (72.1)**	**118 (78.7)**	
Yes	12 (16.2)	8 (24.2)	12 (27.9)	32 (21.3)	
Trimester of pregnancy					0.361
First	5 (6.8)	2 (6.1)	7 (16.3)	14 (9.3)	
Second	23 (31.1)	7 (21.2)	12 (27.9)	42 (28.0)	
Third	46 (62.2)	24 (72.7)	24 (55.8)	94 (62.7)	
Past preterm baby					0.763
No	56 (75.7)	27 (81.8)	34 (79.1)	117 (78.0)	
Yes	18 (24.3)	6 (18.2)	9 (20.9)	33 (22.0)	
Past miscarriage					0.976
No	51 (68.9)	23 (69.7)	29 (67.4)	103 (68.7)	
Yes	23 (31.1)	10 (30.3)	14 (32.6)	47 (31.3)	

Past abortion					1.000
No	71 (95.9)	32 (97.0)	42 (97.7)	145 (96.7)	
Yes	3 (4.1)	1 (3.0)	1 (2.3)	5 (3.3)	
Past abnormal vaginal discharge					0.733
No	40 (54.1)	17 (51.5)	20 (46.5)	77 (51.3)	
Yes	34 (45.9)	16 (48.5)	23 (53.5)	73 (48.7)	
Treated for past sexual infection					0.576
No	48 (64.9)	19 (57.6)	24 (55.8)	91 (60.7)	
Yes	26 (35.1)	14 (42.4)	19 (44.2)	59 (39.3)	

4.3 Culture of *G. vaginalis* from vaginal swabs

Of the 150 vaginal swabs, 16 isolates of *G. vaginalis* were successfully cultured. On the *G. vaginalis* selective plates, the presence of the bacterium was identified by observing zones of clearance around the colonies, as shown in Figure 9. This was indicative of beta-haemolysis. of human erythrocytes, which is characteristic of *G. vaginalis*.

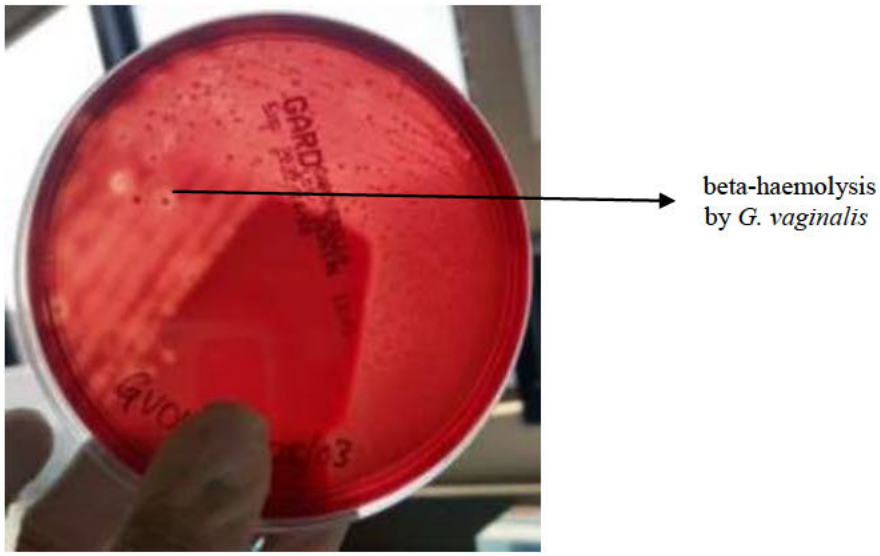


Figure 9: The presence of *G. vaginalis* on selective plates, Beta-haemolysis is shown as zones of clearance around selected colonies.

4.4 Confirmatory assays

Confirmatory tests were conducted on suspected colonies to confirm the presence of *G. vaginalis*. Firstly, Gram-staining of selected colonies revealed the presence of Gram-variable, cocci-bacillus shaped bacteria which were dispersed throughout the slide as shown in Figure 10.

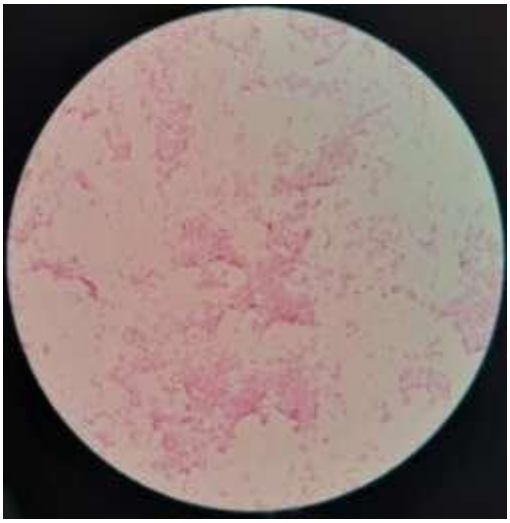


Figure 10: Gram-stain of a *G. vaginalis* isolate under an oil immersion lens.

Biochemical tests (catalase and oxidase tests) were also performed to confirm the identity of *G. vaginalis.* For the catalase test, when a colony was picked and placed into a test tube containing hydrogen peroxide, no reaction occurred (absence of bubbles). For the oxidase test, when a colony was placed onto an oxidase strip, no colour change occurred (strip remained colourless). The 16 isolates were negative for oxidase and catalase as expected (Table 4).

Of the 16 isolates, five isolates were from BV positive women (GV35, GV60, GV62, GV63 and GV138), two were from BV negative women (GV48 and GV49) and nine were from BV intermediate women (GV54, GV55, GV61, GV109, GV113, GV114, GV125, GV129 and GV137) (Table 4).

Table 4: Confirmatory assays for *G. vaginalis* and link between isolates and BV status

Sample	BV status	Catalase	Oxidase
GV35	Positive	Negative	Negative
GV48	Negative	Negative	Negative
GV49	Negative	Negative	Negative
GV54	Intermediate	Negative	Negative
GV55	Intermediate	Negative	Negative
GV60	Positive	Negative	Negative
GV61	Intermediate	Negative	Negative
GV62	Positive	Negative	Negative
GV63	Positive	Negative	Negative
GV109	Intermediate	Negative	Negative
GV113	Intermediate	Negative	Negative
GV114	Intermediate	Negative	Negative
GV125	Intermediate	Negative	Negative
GV129	Intermediate	Negative	Negative
GV137	Intermediate	Negative	Negative
GV138	Positive	Negative	Negative

The isolates were confirmed to be *G. vaginalis* by a PCR-based assay. For this assay, the *16S rRNA* gene, specific for *G. vaginalis,* was used. According to the results, *16S rRNA* was amplified in all 16 isolates, confirming the identity of the isolates. Table 5 shows the amplification data of the real-time PCR assay and the corresponding cycling threshold (Ct)

values for the individual isolates. The negative control was not amplified, and the positive control (ATCC strain) was amplified, validating the PCR results (Table 5).

Table 5: *16S rRNA* gene amplification results with isolate name and Ct value (n=16)

Sample	CT value
Negative control	Undetermined
ATCC	18.959
GV35	14.924
GV48	19.562
GV49	29.513
GV54	18.219
GV55	19.458
GV60	16.971
GV61	20.464
GV62	20.142
GV63	18.167
GV109	19.768
GV113	17.429
GV114	18.589
GV125	18.917
GV129	19.198
GV137	18.470
GV138	18.823

4.5 Genotyping Assays

The genotyping of the isolates was based on genetic variations within the *G. vaginalis tuf* gene. The PCR assay was performed using primer sets specific to the four clades known to be present in *G. vaginalis* strains. Positive amplification was observed for all isolates, and the amplification data for the *tuf* gene is shown in Table 6. According to the analysis, the isolates belonged to clades 1, 2 and 4 as shown in Table 6. There were no isolates that belonged to clade 3. The frequencies for each clade were as follows; 100% for clade 1, 37.5% for clade 2 and 43.75% for clade 4. Multiple clades were found in 25% of the isolates.

Table 6: Assignment of clades for the individual *G. vaginalis* isolates (n=16)

Sample	CT value
Clade 1	
35	31.540
48	18.021
49	31.447
54	18.229
55	18.164
60	15.676
61	34.236
62	24.811
63	20.925
109	30.973
113	16.028
114	16.724
125	17.048
129	17.571
137	16.725
138	17.543
Clade 2	
61	34.124
62	30.204
63	20.085
113	36.830
114	35.279
137	28.558

Clade 4	
48	38.919
60	31.938
61	33.935
62	34.942
109	37.249
129	36.359
137	36.932

4.6 BV status in relation to clades

The link between BV status and *G. vaginalis* clades is shown in Table 7. According to the data, the different *G. vaginalis* clades were distributed across the different BV statuses. No individual clade was responsible for driving a particular BV status. Therefore, there is no substantial link between BV status and *G. vaginalis* clades. We can suggest, that there is a lack of genetic diversity in *G. vaginalis* from the different BV states and that genetics is not driving disease progression.

Table 7: The link between BV status and *G. vaginalis* clades

Isolate	BV status	Clade
35	Positive	1
48	Negative	1, 4
49	Negative	1
54	Intermediate	1
55	Intermediate	1
60	Positive	1, 4
61	Intermediate	1, 2, 4
62	Positive	1, 2, 4
63	Positive	1, 2
109	Intermediate	1, 4
113	Intermediate	1, 2
114	Intermediate	1, 2
125	Intermediate	1
129	Intermediate	1, 4
137	Intermediate	1, 2, 4
138	Positive	1

4.7 Antimicrobial susceptibility testing

In South Africa, only metronidazole is used for the treatment of BV in pregnant women. However, in other parts of the world, clindamycin and vancomycin are also used for the treatment of BV. For antimicrobial susceptibility testing, only 14 isolates were viable for analysis. The MIC value for all isolates were <0.5 µg/ml for clindamycin, therefore all isolates were susceptible to clindamycin. The MIC value for all the isolates were <2µg/ml for vancomycin, thus all isolates were susceptible to vancomycin (Table 8). The resistance of *G. vaginalis* to metronidazole ranged from 2 to 32µg/ml. Of the 14 isolates that were successfully

cultured and tested, 8/14 (57.1%) were susceptible to metronidazole (MIC of ≤8 µg/ml) and 6/14 (42.9%) were resistant to metronidazole (MIC of ≥32 µg/ml) (Table 8 and 9).

Table 8: Antimicrobial susceptibility data for *G. vaginalis* isolates

	MIC (µg/ml)													
ISOLATE	**PEN**	**AMOX**	**AUG2**	**P/T4**	**PIP**	**FOX**	**IMI**	**CHL**	**ERY**	**CLI**	**MRD**	**MXF**	**TET**	**VAN**
ATCC	<0.06	<0.25	<0.25/0.12	<16/4	<16	<0.5	0.12	<2	<1	<0.5	8	0.25	<2	<2
GV35	<0.06	<0.25	<0.25/0.12	<16/4	<16	1	0.12	<2	4	<0.5	32	0.25	4	<2
GV48	0.5	<0.25	<0.25/0.12	<16/4	<16	2	0.5	<2	2	<0.5	8	0.25	<2	<2
GV49	<0.06	<0.25	<0.25/0.12	<16/4	<16	<0.5	<0.06	<2	<1	<0.5	>32	<0.12	8	<2
GV55	<0.06	<0.25	<0.25/0.12	<16/4	<16	<0.5	<0.06	<2	<1	<0.5	>32	<0.12	<2	<2
GV60	0.25	<0.25	<0.25/0.12	<16/4	<16	2	0.5	<2	<1	<0.5	2	0.25	<2	<2
GV61	0.25	<0.25	<0.25/0.12	<16/4	<16	1	<0.06	<2	2	<0.5	8	0.25	<2	<2
GV62	<0.06	<0.25	<0.25/0.12	<16/4	<16	<0.5	<0.06	<2	<1	<0.5	8	0.25	<2	<2
GV63	<0.06	<0.25	<0.25/0/12	<16/4	<16	<0.5	<0.06	<2	<1	<0.5	8	0.25	<2	<2
GV109	0.5	<0.25	<0.25/0.12	<16/4	<16	2	1	<2	<1	<0.5	>32	0.25	<2	<2
GV113	<0.06	<0.25	<0.25/0.12	<16/4	<16	1	0.5	<2	<1	<0.5	8	0.25	>16	<2
GV125	<0.06	<0.25	<0.25/0.12	<16/4	<16	<0.5	<0.06	<2	<1	<0.5	>32	0.5	<2	<2
GV129	0.12	<0.25	<0.25/0.12	<16/4	<16	1	0.25	<2	8	<0.5	8	0.5	<2	<2
GV137	0.25	<0.25	<0.25/0.12	<16/4	<16	2	0.25	<2	<1	<0.5	4	0.5	<2	<2
GV138	<0.06	<0.25	<0.25/0.12	<16/4	<16	2	1	<2	<1	<0.5	>32	1	<2	<2

Table 9: Antimicrobial susceptibility patterns of *G. vaginalis* isolates to metronidazole (n=14)

Isolate	Susceptibility profile
35	Resistant
48	Susceptible
49	Resistant
55	Resistant
60	Susceptible
61	Susceptible
62	Susceptible
63	Susceptible
109	Resistant
113	Susceptible
125	Resistant
129	Susceptible
137	Susceptible
138	Resistant

4.8 Link between BV status, clinical symptoms and metronidazole susceptibility patterns

Table 10 shows the BV states of each isolate and its metronidazole susceptibility profile, as well as clinical symptoms of infection such as abnormal vaginal discharge. Of the *G. vaginalis* isolates cultured from BV positive women (n=5), 60% (3/5) of the isolates were susceptible to metronidazole while 40% (2/5) were resistant. There was no link between resistant patterns and symptoms of infection, since one isolate was from an asymptomatic woman and the other isolate from a symptomatic woman.

Of the *G. vaginalis* isolates cultured from BV intermediate women (n=7), 43% (3/7) of the isolates were resistant to metronidazole while 57% (4/7) were susceptible. There was a link between resistant patterns and symptoms of infection, since all resistant isolates were cultured from symptomatic women. The *G. vaginalis* isolate cultured from the BV-negative women was susceptible to metronidazole and the women were asymptomatic.

Table 10: Link between BV status, clinical symptoms, and metronidazole susceptibility patterns

Isolate	BV status	Susceptibility profile	Clinical symptom (Abnormal discharge)
35	Positive	Resistant	Absent
48	Negative	Susceptible	Absent
49	Negative	Resistant	Present
55	Intermediate	Resistant	Present
60	Positive	Susceptible	Present
61	Intermediate	Susceptible	Absent
62	Positive	Susceptible	Present
63	Positive	Susceptible	Absent
109	Intermediate	Resistant	Present
113	Intermediate	Susceptible	Present
125	Intermediate	Resistant	Present
129	Intermediate	Susceptible	Present
137	Intermediate	Susceptible	Absent
138	Positive	Resistant	Present

4.9 Link between metronidazole susceptibility patterns and clades of *G. vaginalis*

Table 11 shows the link between metronidazole susceptibility patterns and clades of *G. vaginalis*. Of the six resistant isolates, five isolates (83%) were assigned to clade 1 alone. The one remaining resistant isolate was a mixed clade, it was assigned clade 1 and 4. The eight susceptible isolates belonged to mixed clades such as 1 and 2, 1, 2 and 4 and 1 and 4. The resistant isolates were more homogeneous in terms of the clades they belonged. However, it cannot be suggested that a particular clade is driving resistance, since the clades were distributed among susceptible and resistant isolates.

Table 11: Link between metronidazole susceptibility patterns and clades of *G. vaginalis*

Isolate	Susceptibility profile	Clade
35	Resistant	1
48	Susceptible	1, 4
49	Resistant	1
55	Resistant	1
60	Susceptible	1, 4
61	Susceptible	1, 2, 4
62	Susceptible	1, 2, 4
63	Susceptible	1, 2
109	Resistant	1, 4
113	Susceptible	1, 2
125	Resistant	1
129	Susceptible	1, 4
137	Susceptible	1, 2, 4
138	Resistant	1

CHAPTER 5
DISCUSSION

Bacterial vaginosis is characterised by a decrease in hydrogen peroxide producing *Lactobacilli* and an increase in facultative, anaerobic bacteria (Plummer *et al*., 2021). Untreated BV has various negative outcomes, such as increasing the risk of contracting STIs such as HIV, chlamydia, HSV-2, and *Trichomonas vaginalis* (Coudray & Madhivanan, 2020). In this study, the prevalence of BV was 22%. This prevalence is similar to other studies. The reported prevalence of BV during pregnancy ranges from 4.9 to 49% (Ishaque *et al.*, 2011). In a population of pregnant women in South Africa, the prevalence of BV was observed to be 17.6% (Redelinghuys *et al*., 2013). Joyisa *et al*. (2019) conducted a BV prevalence study among HIV uninfected South African pregnant women. Using the Nugent score, 37.3% of the women were BV positive. Another study among pregnant women from Ghana, reported a BV prevalence of 30.9% (Konadu *et al.,* 2019). These high prevalence reports of BV among pregnant women warrant the need for routine screening and effective BV management strategies among pregnant populations.

In the study population, the only variable that was significantly associated with BV status, p=0.025 was being asymptomatic for infection (no current abnormal vaginal discharge). An estimated 50% of the global population is asymptomatic for BV (Mondal *et al*., 2023), thereby confirming the findings of this study. Asymptomatic BV will go untreated and may have serious consequences for pregnant women. In South Africa, women are only treated based on infection symptom; therefore, asymptomatic women can continue to transmit the infection to their sexual partners and unborn babies. Therefore, the implementation of cost-effective

laboratory diagnostics as part of test and treat strategies should be a priority to reduce the burden of this infection.

G. vaginalis is the predominant pathogen responsible for the onset of BV (Pleckaityte *et al*., 2012). According to Li *et al*. (2020), *G. vaginalis* has been isolated in up to 95% cases of BV. The role of *G. vaginalis* has remained a controversy given its notorious presence in both healthy individuals and in women presenting with BV. This finding has led researchers to consider the possibility that there may be different strains within the *Gardnerella* species (Cornego *et al.,* 2018) that possess distinct pathogenicity's that would ultimately produce different clinical outcomes (Janulaitiene *et al.,* 2018). The notion may be that while healthy women are colonised by a less virulent strain of *Gardnerella* species, the development of BV is characterised by more virulent strains (Qin & Xiao, 2022). In this study, of the 150 vaginal swabs, 16 isolates of *G. vaginalis* were successfully cultured from women diagnosed as BV negative, intermediate and positive.

The pathogenic and commensal nature of *G. vaginalis* has led researchers to study the phenotypic and genotypic characteristics of *G. vaginalis* (Ingianni *et al.,* 1997). Extensive bacterial typing assays have been conducted to assess the diversity of *G. vaginalis* based on the biochemical properties and genetic heterogeneity of the strains by biochemical testing and genotyping (Shipitsyna *et al*., 2019). In addition, the study by Ingianni *et al*. (1997), observed that *G. vaginalis* could be separated into at least 4 genotypes. In a study conducted by Schuyler *et al*. (2016), four *G. vaginalis* clades have been identified. The *tuf* gene was amplified in order to group isolates into clades and encodes for the translation of the elongation factor *Tu*. It is also species-specific (Balashov *et al.,* 2014). However, in this study, using clade-specific primers based on the *tuf* gene, three of the four clades of *G. vaginalis* (clade 1, 2 and 4) were detected among the study isolates. The diversity of the *tuf* gene abundance within and between

clades may be indicative of genetic variations or differences in expression of this gene within different *G. vaginalis* populations.

In this study, none of the isolates belonged to clade 3. This was also observed in a study conducted by Morselli *et al*. (2023), in which none of the *G. vaginalis* isolates belonged to clade 3. The lack of isolates belonging to clade 3 is supported by previous work which confirmed that there is a lower occurrence of *G. vaginalis* belonging to clade 3 within the vaginal microbiome (Janulaitiene *et al*., 2018).

Isolates have found to belong to only one clade. In this study, clade 1 was the dominant clade. In a study conducted by Severgnini. *et al*. (2022), 18.9% of *G. vaginalis* isolates were reported to be of one particular clade. This study investigated the link between the status of BV status and the *G. vaginalis* clades. According to the data, the different clades of *G. vaginalis* clades were distributed across the different states of BV. Additionally, some of the isolates in our study belonged to more than one clade. This is supported by Plummer *et al*. (2019), who reported that 65.19% of their study isolates belonged to multiple clades. This was evident in the isolates from intermediate women with BV. Our findings differ from previous work that reported the presence of multiple clades in women with a BV positive status only (Severgnini *et al*., 2022). In this study, clade 2 was only detected in multiclade communities. A similar observation was reported by Janulaitiene *et al*. (2017).

A previous study found that clades 1 and 4 are often associated with positive BV (Qin *et al*., 2023). However, based on the observation our study, we found that no individual clade was responsible for driving a particular BV status. Therefore, there is no substantial link between BV status and *G. vaginalis* clades. As mentioned previously, we can suggest, that there is a lack of genetic diversity in *G. vaginalis* from the different BV states and that bacterial genetics is not driving disease progression.

Currently, BV is treated with broad-spectrum antibiotics such as metronidazole, clindamycin, and vancomycin (Bradshaw & Sobel, 2016). These antimicrobials are administered orally or in cream form (Nagaraja, 2008). In South Africa, the most commonly used antibiotic for symptomatic BV is metronidazole (Joyisa *et al*., 2019). In this study, antimicrobial susceptibility patterns to metronidazole, clindamycin and vancomycin were investigated. Isolates were susceptible to clindamycin and vancomycin. However, 42.9% of the isolates were resistant to metronidazole. Previous studies have shown that there are strains of *G. vaginalis* that are resistant to metronidazole (Jones *et al*., 2019). Similarly, in a study conducted by Li *et al*. (2020), it was concluded that *G. vaginalis* was highly tolerant to metronidazole. Currently, in South Africa, metronidazole is used only for the treatment of BV. This emerging resistance raises concerns and advocates for the licensure of the use of clindamycin and vancomycin in South Africa, provided that it is safe to use during pregnancy. In this study, of the *G. vaginalis* isolates cultured from BV positive women, the majority of the isolates were susceptible to metronidazole while 40% were resistant. There was no link between resistant patterns and infection symptoms, as one resistant isolate was from an asymptomatic woman and the other resistant isolate was from a symptomatic woman. However, for the BV intermediate women, there was a link between resistant patterns and infection symptoms, since all resistant isolates were cultured from symptomatic women. This suggests a potential association between an intermediate BV status and the presence of abnormal discharge. Previous studies have shown that untreated BV may lead to various negative outcomes for both mother and baby, as well as the overall reproductive health of the infected individual (Bhakta *et al*., 2021). This study showed that women with a BV intermediate status have been experiencing abnormal vaginal discharge. It is likely that these women were being treated for abnormal discharge. However, *G. vaginalis* isolates from symptomatic women were resistant to metronidazole and it is likely that these women were failing treatment. Based on this, these women may continue to

experience abnormal discharge and their BV intermediate status may likely progress to a BV positive status that will be untreatable since they are already resistant.

This study also investigated the link between metronidazole susceptibility patterns and *G. vaginalis* clades. The resistant isolates were more homogeneous in terms of the clades to which they belonged to, with clade 1 being the predominant clade. However, our findings differ to that of Morselli *et al*. (2023), who reported that the highest frequency of metronidazole resistance was found in clade 2, followed by clade 1 then clade 4. Another study by Qin *et al*. (2023), confirmed that *G. vaginalis* clade 3 and clade 4 strains were previously shown to have 100% resistance to metronidazole. However, based on the findings of our study, it cannot be suggested that a particular clade is driving resistance as the clades were distributed across susceptible and resistant isolates.

The limitations of the study were as follows.

This study had a small sample of isolates, only 16 isolates were analysed. To conduct antimicrobial resistance surveillance and diversity assessments, a larger sample of isolates would need to be analysed.

This study did not investigate the presence of antimicrobial resistant determinants however, future research can focus on sequencing the genomes of these isolates in order to identify the full cascade of resistant determinants.

This study only focused on isolates cultured from pregnant women, and, for future studies on resistance surveillance and diversity assessments, a more general population of women should be investigated.

CHAPTER 6
CONCLUSION

The high prevalence of BV in the studied population warrants the need for routine screening and management of BV to reduce the burden of this dysbiosis on women's sexual health. Based on diversity assessments conducted, clade 1 was shown to be the predominant clade across isolates. However, when comparing clades to the BV status, this study found that there was no substantial link between the BV status and clades of *G. vaginalis.* We therefore suggest that bacterial genetics is not driving disease progression in the case of BV; however, in order to provide sound conclusions a larger sample of isolates would need to be analysed, as well as isolates obtained from a more general population of women.

In this study, 40% of *G. vaginalis* isolates were resistant to metronidazole. As discussed previously, in South Africa, metronidazole is only used for the treatment of BV. This emerging resistance raises concerns and advocates for the licensure of the use of clindamycin and vancomycin in South Africa, provided that it is safe to use during pregnancy. Furthermore, this study found that the *G. vaginalis* isolates from the symptomatic BV intermediate women were resistant to metronidazole and it is likely that these women were failing treatment. Based on this, these women may continue to experience abnormal discharge and their BV intermediate status may likely progress to a BV positive status which will be untreatable since they are already resistant. Therefore, the testing of newer antimicrobials for BV is of public health importance.

CHAPTER 7

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CHAPTER 8

Appendices

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

06 July 2023

Miss Thasmika Durga (218030980)
School of Clinical Medicine
Medical School

Dear Miss Durga,

Protocol reference number: BREC/00005700/2023
Project title: Investigating antimicrobial resistance patterns in Gardnerella vaginalis isolates
Degree: MMedSc

EXPEDITED APPLICATION: APPROVAL LETTER

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application.

The conditions have been met and the study is given full ethics approval and may begin as from 06 July 2023. Please ensure that any outstanding site permissions are obtained and forwarded to BREC for approval before commencing research at a site.

This approval is valid for one year from 06 July 2023. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on RIG on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2020) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be noted by a full Committee at its next meeting taking place on 11 July 2023.

Yours sincerely,

[redacted]

Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

Biomedical Research Ethics Committee
Chair: Professor D R Wassenaar
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