Genotyping of *Gardnerella vaginalis* from pregnant women in Durban by Amplified Ribosomal DNA Restriction Analysis

By Kayla Pillay

Student number: 219090946

Supervisor: Dr Nathlee Abbai

Co-supervisor: Dr Meleshni Naicker

June 2020

Submitted in partial fulfilment of the requirements for the Degree of Master of Medical Science

in the School of Clinical Medicine, College of Health Sciences

University of KwaZulu-Natal

Durban

South Africa

DECLARATION

I, **Kayla Pillay**, declare that

i. The research reported in this dissertation, except where otherwise indicated, is my original work.

ii. This dissertation has not been submitted for any degree or examination at any other university.

iii. This dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

iv. This dissertation does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
   a. their words have been re-written but the general information attributed to them has been referenced.
   b. where their exact words have been used, their writing has been placed inside quotation marks, and referenced.

v. Where I have reproduced a publication of which I am an author, co-author or editor, I have indicated in detail which part of the publication was actually written by myself alone and have fully referenced such publications.

vi. This dissertation does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the dissertation and in the References sections.

Signed: 

Date: 01 June 2020

PERMISSION TO SUBMIT

As the candidate's supervisors, I have read the thesis and have given approval for submission for examination

[redacted]

Supervisor: Dr Nathlee Abbai

Date: 12 June 2020

[redacted]

Co-supervisor: Dr Meleshni Naicker

Date: 12 June 2020

Discipline: Department of Medicine

School of Clinical Medicine

College of Health Sciences

University of KwaZulu-Natal South

Africa

ACKNOWLEDGEMENTS

I would like to thank God for all the spiritual guidance I have received throughout my journey, and for blessing me with ambition and determination to pursue my dreams.

My most sincere gratitude is extended to:

1. Dr. Nathlee Abbai, my supervisor, for her continued support and guidance, patience, motivation, research expertise, and assistance in the design and execution of this study.

2. Dr. Meleshni Naicker, my co-supervisor, for mentoring, supporting, and encouraging me, and for her assistance in the practical aspect of my study as well as in the interpretation of my results and compilation of my dissertation.

3. Mrs Fazana Dessai, Laboratory Manager of the Clinical Medicine Laboratory, for her assistance in and around the laboratory.

4. Dr. Partson Tinarwo, Department of Biostatistics, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, for conducting the biostatistics analysis.

5. A host of family and friends, whom have always motivated me to be my best. For their continued support, love, patience, and contributions throughout the duration of this degree. I am truly appreciative to every single one of you.

ACCEPTED MANUSCRIPT

- Pillay, K., Nzimande, S., Naicker, M., Ramsuran, V., Tinarwo, P. and Abbai, N. 2020. Prevalence of genotypes and subtypes of *Gardnerella vaginalis* in South African pregnant women. *Accepted in Infectious Diseases in Obstetrics and Gynecology, (June, 2020).*

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

LIST OF ABBREVIATIONS AND ACRONYMS	
ARDRA	Amplified Ribosomal DNA Restriction Analysis
BLAST	Basic Local Alignment Search Tool
BREC	Biomedical Research Ethics Committee
BV	*Bacterial Vaginosis*
CDC	Cholesterol-Dependent Cytolysin
DNA	Deoxyribonucleic Acid
GV	*Gardnerella vaginalis*
HIV	Human Immunodeficiency Virus
HSV-2	Herpes Simplex Virus-2
MEGA	Molecular Evolutionary Genetics Analysis
NAD	Nicotinamide Adenine Dinucleotide
NCBI	National Centre for Biotechnology Information
NS	Nugent Score
PCR	Polymerase Chain Reaction
PID	Pelvic Inflammatory Disease
REA	Restriction Enzyme Analysis
RFLP	Restriction Fragment Length Polymorphism
rRNA	Ribosomal Ribonucleic Acid
STD	Sexually Transmitted Disease
STI	Sexually Transmitted Infection
TCA	Tricarboxylic Acid

ABSTRACT

Gardnerella vaginalis is one of the most frequently isolated microorganisms associated with bacterial vaginosis (BV). However, limited information concerning the genetic diversity of *G. vaginalis* isolated from BV positive and intermediate cases, has been documented. This study investigated the diversity of *G. vaginalis* in pregnant women, a currently under-researched area in South Africa. The study population included pregnant women recruited from a public hospital in Durban, South Africa. The women provided 2 self-collected vaginal swabs for microscopy and the genotyping assays. The BV status of the women was determined using Nugent scoring. A total sample of n=137 specimens was selected for analysis. The 16S ribosomal ribonucleic acid *(*rRNA*)* gene of *G. vaginalis* was used for the genotyping assays. The 16S rRNA gene polymerase chain reaction products were digested with *TaqI* to generate genotyping profiles and genotypic subtypes were determined by correlating *BamHI* and *HindIII* digestion profiles. Phylogenetic analysis was performed on the 16S rRNA gene sequences. The data analysis was performed in R Statistical Computing software, version 3.6.2. Restriction digestion with *Taq*I revealed the presence of two different genotypes i.e. GT1 and GT2. Within both BV positive and intermediate sample groups, GT1 was the most prevalent genotype (54%). Overall, 4 subtypes (1, 2B, 2AB and C) were shown to be present in the sample population. The most prevalent subtype was 2B (15/37, 40.5%), followed by subtypes 1 (11/37, 29.7%), 2C (4/37, 10.8%) and 2AB (4/37, 10.8%). The phylogenetic analysis of the 16S rRNA genes showed the presence of 5 clusters. The tree displayed clusters which contained groups of specimens from the same BV group with different genotypes and subtypes present. There were also clusters which contained specimens from across the BV groups carrying the

same genotype and subtype. Finally, the study did not find a significant association ($p>0.05$) between reported symptoms of discharge and genotype harboured.

This study provides the first report on the diversity of *G. vaginalis* in South African pregnant women. Diversity assessments of *G. vaginalis* with respect to genotypes and subtypes may aid in a greater understanding on the pathogenesis of this microorganism.

CHAPTER 1

INTRODUCTION

Bacterial Vaginosis (BV) is a common female reproductive infection caused by disruptions in the vaginal microflora when native *lactobacilli* are replaced by anaerobic bacteria [1, 2]. Infection is characterised by vaginal discharge which is foul smelling [3], and irritation during urination [4]. However, some infections may be asymptomatic [5]. The epidemiology of BV documented in previous studies strongly indicates that it is acquired via sexual transmission [6, 7]. Therefore, the causes of BV may be attributed to having multiple sex partners [8] and lack of condom use. Consequences of BV include: adverse pregnancy outcomes [9] and increased susceptibility to sexually transmitted infections (STIs) [10] as well as Human Immunodeficiency Virus (HIV) [11].

The predominant, or aetiological agent, *Gardnerella vaginalis* [4, 12], is found in most women with vaginosis and has been reported to be the main cause of clinical signs and symptoms used to diagnose BV [13, 14]. Other diverse anaerobic bacteria such as *Atopobium*, *Mobiluncus*, *Prevotella*, *Bacteroides*, *Anaerococcus*, *Peptostreptococcus*, *Sneathia*, *Leptotrichia*, and members of the class *Clostridia* [13], are believed to be associated with BV [15]. *G. vaginalis* has appeared to be the most virulent BV-associated anaerobe demonstrating greater adherence to vaginal cells, cytotoxicity, and biofilm-producing capacity [16].

For over three decades, researchers have been conducting extensive bacterial typing assays, in order to identify different virulence traits among *Gardnerella* spp. [17]. Phenotypic assays have been used to assess the diversity of *Gardnerella* spp. based on their biochemical properties such as production of β-galactosidase, lipase, and hippurate hydrolysis.

However, the early typing assays had failed to reveal the diversity of *G. vaginalis* [14, 18]. The genetic heterogeneity of *G. vaginalis* species has been determined using molecular approaches, such as Amplified Ribosomal DNA restriction analysis (ARDRA) [19]. ARDRA is a simple, fast and reproducible method for microbial molecular epidemiology and taxonomy [20]. The ARDRA genotyping approach developed by Ingianni and co-workers was shown to be less error-prone [21]. In the study by Ingianni *et al.,* 1997 [22], the ARDRA method allowed for *G. vaginalis* to be separated into at least 4 genotypes.

Despite the availability of useful genotyping techniques for *G. vaginalis*, it has been documented that there is limited data on the prevalence of *G. vaginalis* genotypes from across the globe [23]. Thus, this study investigated the diversity of *G. vaginalis* from non-cultured vaginal swabs obtained from pregnant women from KwaZulu-Natal, South Africa, by ARDRA, thereby filling in a gap in knowledge. In addition, no clear association between BV and any of the ARDRA genotypes has been reported. Through this study, the distribution of *G. vaginalis* ARDRA genotypes linked to BV status and clinical symptoms of BV such as abnormal vaginal discharge will be determined.

The objectives of the study: to determine the presence of *G. vaginalis* in study samples, to determine the different genotypes that are present across the BV states, and to investigate the association of identified genotypes and/or subtypes with clinical symptoms, all assist in the accomplishment of the overall aim of the study, i.e. to determine the diversity of *Gardnerella vaginalis* in pregnant women from Durban, by identifying different genotypes using Amplified ribosomal DNA restriction analysis (ARDRA).

CHAPTER 2

LITERATURE REVIEW

2.1. Background

Bacterial vaginosis (BV) is an imbalance of the vaginal micro-environment [24], common in females of a reproductive age [2]. Symptoms of BV include: a malodourous white/ grey vaginal discharge, raised vaginal pH (>4.5), burning sensations during urination, vaginal irritation or discomfort. In addition, BV is often asymptomatic [5]. BV is the most common vaginal infection in women of reproductive age [25]. Untreated BV has led to conditions such as pelvic inflammatory disease, increased risk of preterm birth, greater susceptibility to STIs, and neonatal complications [26]. Some of the risk factors for BV include: having a new or multiple sex partners [8], antibiotics, and the use of intrauterine devices. The pathogen or aetiological agent predominantly responsible for the onset of BV infection is *Gardnerella vaginalis*, a facultative anaerobic coccobacillus, also implicated in male and female urogenital tract infections, and bacteraemia [27]. Its pathogenesis is attributed to its production of sialidase A, and the toxin vaginolysin, together with its ability to adhere to vaginal epithelial cells and establish biofilms [16, 28].

2.2. Epidemiology of BV

The prevalence of BV has been reported to be as high as 51% in African women [29]. In a study conducted by Kenyon *et al.*, 2013 [30] which describes the global epidemiology of BV, it was observed that the prevalence of BV varies due to ethnicity and geographic regions.

According to that study, BV prevalence was the highest in Southern and Eastern Africa.

According to a study by Bayigga *et al.*, 2018 [31], BV prevalence among women in sub-Saharan Africa is approximately 40% [32, 33], with reports of the condition persisting despite available treatment. In Africa, South Africa (SA) had the highest prevalence of BV [34]. Gambia had the second highest prevalence of BV at 37% followed by Uganda at 34%. The lowest prevalence of BV was 12% in Maputo, Mozambique [30].

KwaZulu-Natal (KZN), the most densely populated province in SA, has been markedly affected by both the HIV and STI epidemic [35], with a disproportionate burden of STIs and HIV among women. Several studies conducted with various populations of women in KZN have shown high prevalence of HIV coupled with STIs [36-38]. In Durban, KZN, the prevalence of BV was 52% and in rural KZN the prevalence of BV was around 58%.

2.3. Risk factors for BV

Epidemiological studies revealed that risk factors for the acquisition of BV include low socio-economic status, cigarette smoking, douching, antibiotic treatment for other conditions, and the acquisition of new or multiple sex partners [39-44], amongst others. These high-risk behaviours are also linked to the acquisition of common STIs, emphasising that BV could be transmitted sexually [41, 45-47].

Studies in a Ugandan population of women at high risk of acquiring HIV/STIs showed that the high prevalence of BV was attributed to women being of a young age, the consumption of alcohol, and the number of previous partners had [48]. The association of bacterial STIs and BV incidence in Durban has also been studied [10]. According to Nagot *et al.*, 2007 [49], Herpes Simplex Virus-2 (HSV-2) infected women have a higher prevalence of BV when compared to uninfected women. It has been previously shown that women diagnosed as HSV-2 positive, older than 35 years of age, not married, having a partner who was not circumcised

and coital frequency in the last 7 days were all associated with increased likelihood of BV incidence [50].

2.4. Consequences of BV during pregnancy

Of particular public health concern is the observation that BV is the most commonly reported microbiological syndrome among women of childbearing age [4, 25]. BV can be an independent risk factor for the acquisition of many other STIs [51], commonly HIV. Adverse pregnancy outcomes associated with BV as depicted in Figure 1 include: infertility, high prenatal mortality, preterm birth, postpartum fever, development of endometritis, post-hysterectomy, or post-abortal sepsis, other post-surgery infections, upper genital tract infections, and pelvic inflammatory disease [15, 52, 53] have attracted the attention of both clinicians and the scientific community at large [54]. In addition to female reproductive health, foetal health is also compromised upon infection with BV. BV-associated microorganisms and their toxins capable of crossing the placenta are among the main causes of brain injury for foetuses, with long-term neurological consequences such as hyperactivity, academic difficulties, and more severely, cerebral palsy, observed in children [55-57].

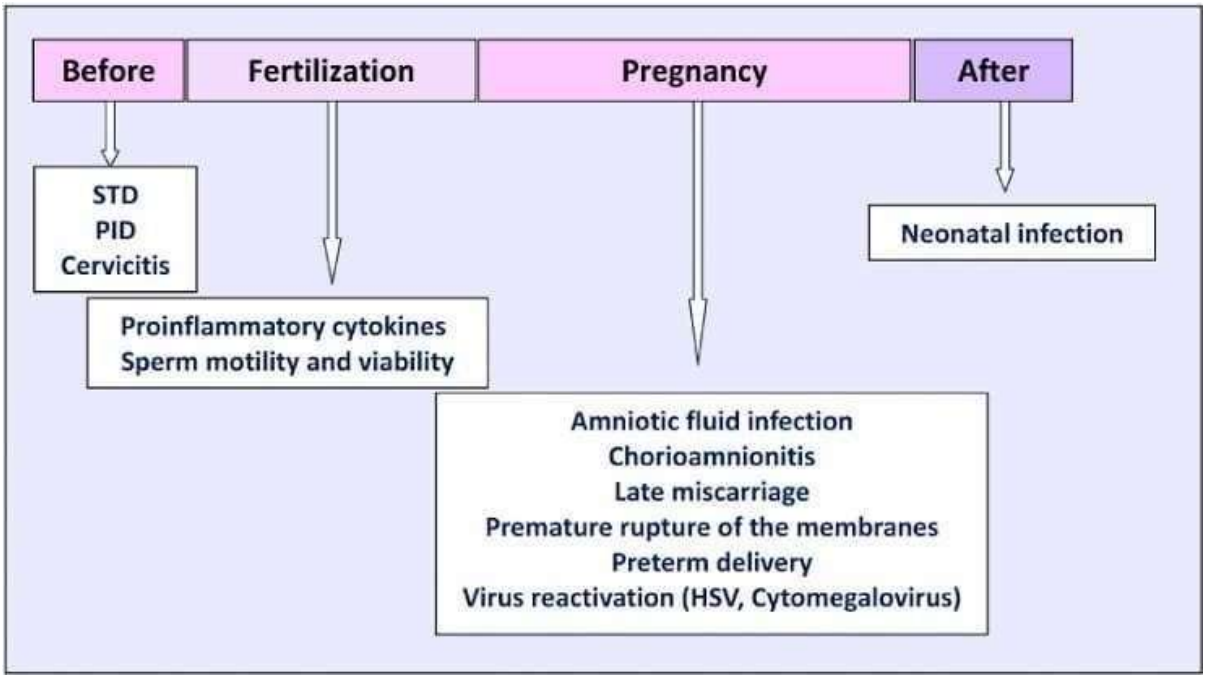


Figure 1: Implications of bacterial vaginosis on reproductive health. The figure depicts maternal and neonatal complications associated with bacterial vaginosis infection at various stages i.e. pre-pregnancy, during pregnancy and post-pregnancy. STD, sexually transmitted disease, PID, pelvic inflammatory disease. Image taken from Mastromarino et al., 2014 [58].

2.5. Diagnosis of BV

Due to its notorious ability for setting off an entire array of serious gynaecological and obstetric complications [12], and its controversial presence in women presenting with and without clinical symptoms, there is an increased need for accurate BV diagnosis.

2.5.1. Clinical methods

Saline microscopy is the most recognised method for clinical diagnosis of BV. Due to the sensitivity and specificity rates (ranging from 37% to 70% and 94% to 99%, respectively) [59, 60], Amsel's criteria is mostly used for diagnosis of BV. A homogenous discharge, elevated vaginal pH , 4.5, 20% of clue cells on saline microscopy; and a fishy odour after the addition of 10% potassium hydroxide to a slide of secretions (positive whiff test) [3] serve as the criteria

for diagnosis, of which three of the four must be met. The adherence of *G. vaginalis*, with increased ammonium production, resulting in the production of cytotoxins and an exfoliated biofilm leading to appearance of clue cells, is facilitated by the rise in pH that occurs in BV. This enhances the growth and establishment of other BV-associated anaerobes as depicted in Figure 2.

A relatively fast and convenient diagnosis using microscopy can be made, thus proving to be a desired method for some clinicians. However, shortcomings of this method include the observation that routine assessment of all criteria is sometimes not carried out, and diagnosis of BV is inaccurate by clinicians due to lack of time or skills [61].

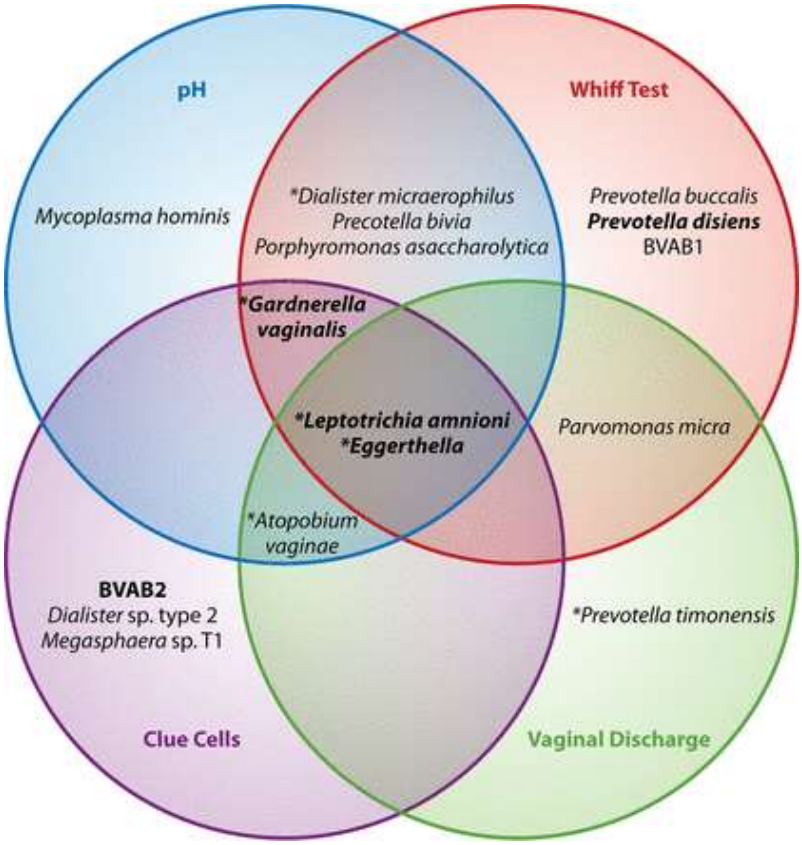


Figure 2: Relationship between Amsel's criteria and bacterial communities in women with bacterial vaginosis. Represented are the different bacterial taxa associated with significant factors used in the diagnosis of bacterial vaginosis according to the Amsel's criteria [15]. For example, *Gardnerella vaginalis* is shown to be associated with increased pH, clue cells, and a positive whiff test. Asterisks represent bacteria present in 75% of women with bacterial vaginosis, and taxa in bold font are those associated with Amsel's criteria as a composite unit. Image taken from Coleman and Gaydos, 2018 [62].

2.5.2. Laboratory-based methods

The Gram stain is the 'Gold-standard' for the diagnosis of BV [63], and has been used in laboratories since 1965 [62]. This method is largely based on the presence or absence of *lactobacilli*.

The use of microscopy on gram stained vaginal smears, together with a standardised scoring system for BV, the Nugent score (NS), is used to distinguish the three bacterial morphotypes with the highest degree of reproducibility: *Lactobacillus* (large Gram-positive rods), *Gardnerella* and *Bacteroides* spp. (small Gram positive or Gram-variable rods), and *Mobiluncus* spp. (curved Gram-negative or Gram variable rods). With the Nugent score, the slides are examined for the quantity of Gram-positive rods and *lactobacilli* (i.e., normal flora) and Gram-negative or Gram variable morphotypes (BV flora) [64]. A score of 0 to 3 indicates 'normal flora', 4 to 6, 'intermediate or mixed flora', and 7 to 10 is indicative of a positive BV status [64, 65].

2.6. History of *G. vaginalis*

BV was initially thought to be a STI propagated by a bacterium that is now known as *G. vaginalis*. Although the discovery of this pathogen was made in 1953 by Leopold, the description of the microorganism in relation to BV was documented by Gardner and Dukes in 1955. Controversy surrounds the history of this microorganism as its elusive nature is even revealed by the fact that it has been renamed several times, mainly because of its unique cell wall structure and nutritional requirements [12].

Leopold [66] originally described this microorganism as a novel "*Haemophilus*-like" species associated with prostatitis and cervicitis [67]. The classification of the microorganism as *Haemophilus vaginalis*, based on its origin, was validated by its cell morphology, then-negative reaction to Gram staining, and its inability to grow on agar media lacking blood. Eventually, its Gram-variable nature differentiated it from other members of the *Haemophilus* genus when it was realised that *Haemophilus vaginalis* occasionally had a positive reaction to Gram staining and did not require either hemin or Nicotinamide Adenine Dinucleotide (NAD) for its growth. The microorganism was then temporarily placed into the *Corynebacterium* genus, and for some

time was referred to as *Corynebacterium vaginale* by Zinnemann and Turner [68, 69]. However, its biochemical profile did not fit into the description of the *Corynebacterium* genus as the microorganism was catalase-negative, and lacked arabinose in its cell wall [12, 67]. Finally, two large taxonomic studies evaluating multiple criteria revealed the lack of similarities between "*Haemophilus vaginalis*" and other established genera [70, 71]. As a result, a new genus named *Gardnerella* was proposed, with *G. vaginalis* being the only species in it.

As the polymicrobial nature of BV became evident, the role of *G. vaginalis* in the aetiology of this condition became less clear. As a result, interest in *G. vaginalis* declined in the late 1980s, only to re-emerge in recent years as the relationship of this microorganism to BV was re-evaluated.

2.7. Structure and physiology

Numerous attempts to study the biochemistry and ultrastructure of the *G. vaginalis* cell wall have led to some conflicting results [4]. Due to the relatively thin cell wall (8-12nm) encompassing a thin peptidoglycan layer, *G. vaginalis* is classified as Gram-variable [72] based on its reaction to Gram staining as it varies from negative to positive [67]. *G. vaginalis* is a facultative anaerobe with cells that are typically small, pleomorphic coccobacilli (0-4 by 1-1.5μm) [67] as shown in Figure 3, ranging from 2-3μm in length [70, 73]. The cells are immotile, non-sporulating, uncapsulated, and lack flagella [73]. Additionally, they possess fimbriae (3-7.5nm in diameter) [63], that functions together with exopolysaccharides to assist in the attachment of *G. vaginalis* to vaginal epithelial cells *in vivo* [67, 74]. The presence of pili is more frequent in clinically isolated strains compared to laboratory cultured strains [75].

Cells frequently occur in clumps in vaginal smears and when grown in liquid media [70]. Cell size and morphology is largely dependent on growth conditions and physiological state.

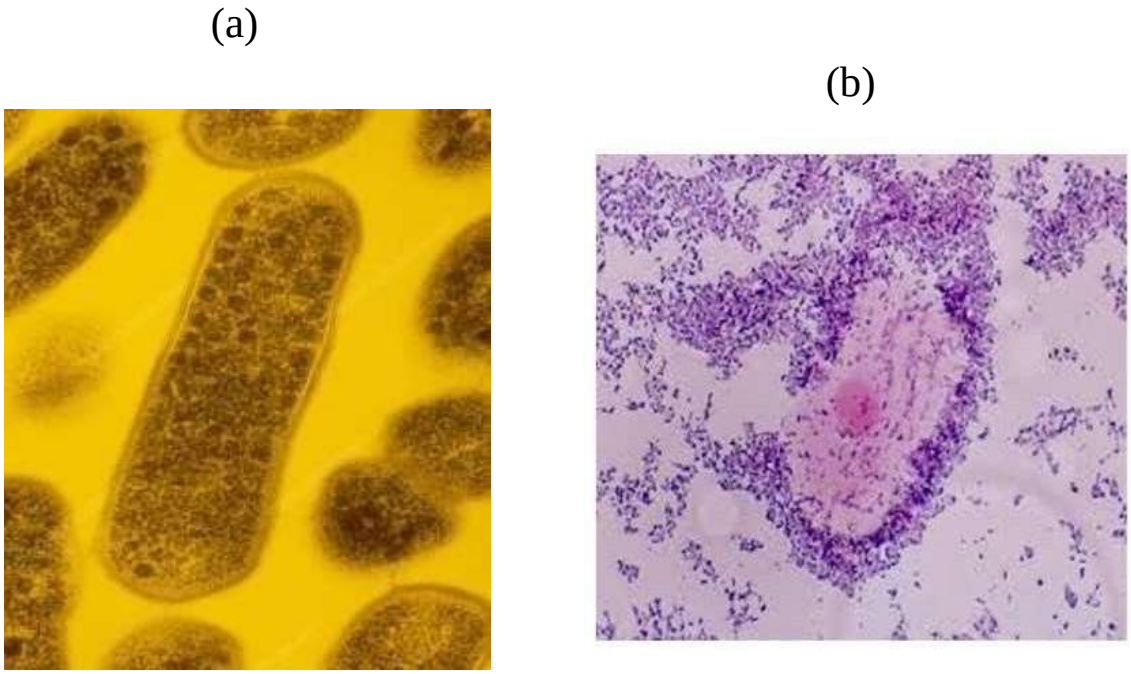


Figure 3: (a) Structure of the pleomorphic coccobacillus *Gardnerella vaginalis*. (b) Gram stain of *Gardnerella vaginalis* as seen under a microscope. Image taken from Centre for Disease Control and Prevention, 2010.

2.8. Nutrient/growth requirements and metabolism

G. vaginalis is a fastidious, facultative anaerobe that grows well in microaerophilic conditions in 5-7% carbon dioxide [76]. Optimum growth of *G. vaginalis* occurs between pH 6 and 6.5 and temperatures between 35^{o}C and 37^{o}C. Slight growth occurs between pH 4.5 and 8 and temperatures between 25^{o}C and 45^{o}C, and little or no growth occurs at pH 4 [70, 72]. It does not require either hemin or NAD for growth. Biochemically, *G. vaginalis* is catalase- [12], oxidase- and β-glucosidase negative [67]. This microorganism ferments a wide range of carbohydrates including starch, dextrin, sucrose, glucose, fructose, ribose, maltose and

raffinose, resulting in the production of acetic acid as the major end product [77]. Some strains can also ferment xylose and trehalose. Conversely, *G. vaginalis* is unable to ferment rhamnose, melibiose, mannitol, and sorbitol [78]. Additionally, *G. vaginalis* can hydrolyze hippurate but not gelatin or esculin. This microorganism is also positive for α-glucosidase activity and for β-hemolysis on human blood, but not sheep's blood [12].

Findings regarding some of the complex nutritional requirements that are needed for *in vitro* growth of *G. vaginalis* were explained. *G. vaginalis* inhabits an environment in which a number of potential iron sources may be available. Interestingly, the analysis of *G. vaginalis*' genome revealed that, aside from simple conversions, the microorganism lacks enzymes in biochemical pathways involved in amino acid synthesis [12]. It was predicted that *G. vaginalis* can synthesise some but not all purine and pyrimidine bases [78].

The acquisition of iron by microorganisms plays a crucial role in the growth of most pathogens [79]. In a study conducted by Jarosik *et al.,* 1998 [80], the ability of *G. vaginalis* strains to acquire iron from various media, and the mechanisms of iron acquisition was explored. In plate bioassays, the studied strains were able to acquire iron from ferric and ferrous-, hemin, catalase, and haemoglobin substrates, as well as lactoferrin, but not transferrin, of several mammalian iron sources *in vitro* [79] as shown in Figure 4. Lactoferrin [81], an extracellular iron-binding glycoprotein, can be found on mucosal surfaces, including those of the urogenital tract. Intracellular haemoglobin is found in erythrocytes and is presumably released upon lysis of erythrocytes by the *G. vaginalis* hemolysin [82]. The results from the study revealed the production of siderophores by *G. vaginalis*, the suggestion of one mechanism by which the microorganism can acquire iron. *G. vaginalis* was also able to directly bind iron-containing compounds such as heme and catalase, indicating that *G. vaginalis* also uses a direct binding mechanism in addition to a siderophore-mediated mechanism to obtain iron [80]. In

conjunction with other studies, *G. vaginalis* is believed to possess up to three potential mechanisms for iron uptake. The glycolytic pathway, seemingly deficient in *G. vaginalis*, was compensated for by the identification of the enzymes responsible for portions of the pentose phosphate pathway. In addition, majority of the genes coding for enzymes involved in the tricarboxylic acid (TCA) cycle are missing from the *G. vaginalis* genome, as reported by Yeoman *et al.*, 2010 [83].

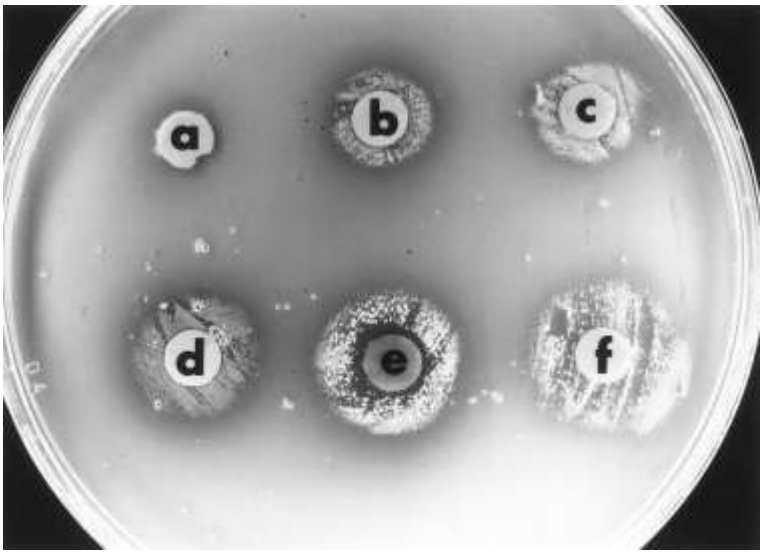


Figure 4: Utilisation of iron sources by *Gardnerella vaginalis* 594 as determined by the plate bioassay. *G. vaginalis* cells were inoculated onto a peptone-starch-dextrose plate containing 100μM deferoxamine mesylate. Filter discs were spotted with distilled water (a), ferric chloride (b), ferrous chloride (c), catalase (d), bovine hemin (e), or bovine haemoglobin (f). Image taken from Jarosik et al., 1998 [80].

The vaginal environment is altered by an increased pH resulting from the decline in *Lactobacillus* responsible for producing hydrogen peroxide [84]. The depletion of native *lactobacilli* results in a significant polymicrobial shift, promoting growth conditions for *G.*

vaginalis and other opportunistic anaerobes [85, 21]. Additionally, a reduction in redox potential by *G. vaginalis* also permits the overgrowth of strict anaerobes.

2.9. Transmission and Pathogenesis

As reviewed elsewhere [7, 6], the epidemiology of BV strongly indicates that it is acquired via sexual transmission. The presence of *G. vaginalis* in healthy women casts doubt on its virulence potential [86, 87]. Nevertheless, in the past decade, it has been demonstrated that *G. vaginalis* had a significantly higher virulence potential than many other BV-associated species [16, 88, 89]. Possible virulence factors that could elucidate the pathogenic potential and possible role of *G. vaginalis* in BV, have been considered [4].

G. vaginalis as the aetiological agent of BV, possesses a number of molecular characteristics and employs several mechanisms that can lead to the development of disease. The initial steps for establishing infection include: adherence to host receptor sites [78], production of cytotoxic substances specific for host cells, and biofilm formation, as shown in Figure 5. The difference in cytotoxicity among *G. vaginalis* strains is attributed to their ability to adhere and form a biofilm [16, 78]. The biofilm is critical for the survival of *G. vaginalis*. BV is demonstrated to be a biofilm mass composed predominantly of *G. vaginalis* [90]. The biofilm assists in the adherence of *G. vaginalis* to vaginal epithelial cells, and in the establishment of other BV signature bacteria such as *Atopobium vaginae* in the vaginal microbiome [91]. The production of the biofilm increases the tolerance of *G. vaginalis* to lactic acid and hydrogen peroxide by *lactobacilli*, resistance to antimicrobial treatment (heightened antibiotic tolerance) [92], and resistance to host immune defences [93], resulting in the recurrence of the BV syndrome.

The haemolytic activity of *G. vaginalis* is attributed to the production of vaginolysin, found in all *G. vaginalis* strains so far and considered to be the main and best characterised virulence

factor of *G. vaginalis* [94, 95]. It is a cholesterol-dependent cytolysin (CDC), species-specific for human cells (particularly erythrocytes [94], neutrophils, and endothelial cells), that encodes a pore-forming toxin which binds to the CD59 human complement regulatory molecule [95]. This cytotoxin assists in the initial adherence of *G. vaginalis* to the host epithelial cells and as a cytolysin, it activates the protein kinase pathway in epithelial cells and causes cell death by lysing specific human cells. *In vivo*, this increases nutrient availability for *G. vaginalis* [83].

The pathogenetic mechanism of *G. vaginalis* involves the multiplication of invading bacteria and the establishment of the biofilm community in order to secure survival [90]. Sialidase also known as neuraminidase [96], only produced by certain strains of *G. vaginalis*, functions to destroy the protective mucus layer on the vaginal epithelium by hydrolysing sialic acid on the glycans of mucous membranes, believed to be important in BV [18, 83]. This process assists in the adhesion of bacterial cells on the epithelium through the enhancement of biofilm production (mucinase activity) [97]. Sialic acid also aids in immune response and provides a source of nutrition [28, 98].

In addition to sialidase, *G. vaginalis* and other BV-associated bacteria express prolidases [99]. These proteolytic enzymes also assist in the degradation of extracellular components such as mucin, and are largely associated with BV [14]. Therefore, compromised host immunity [92, 93], epithelial cell uptake, biofilm-forming capacity [16, 90], and metabolic activities of *G. vaginalis* contribute to the survival, diversity and resilience of BV- associated microbiota to therapy.

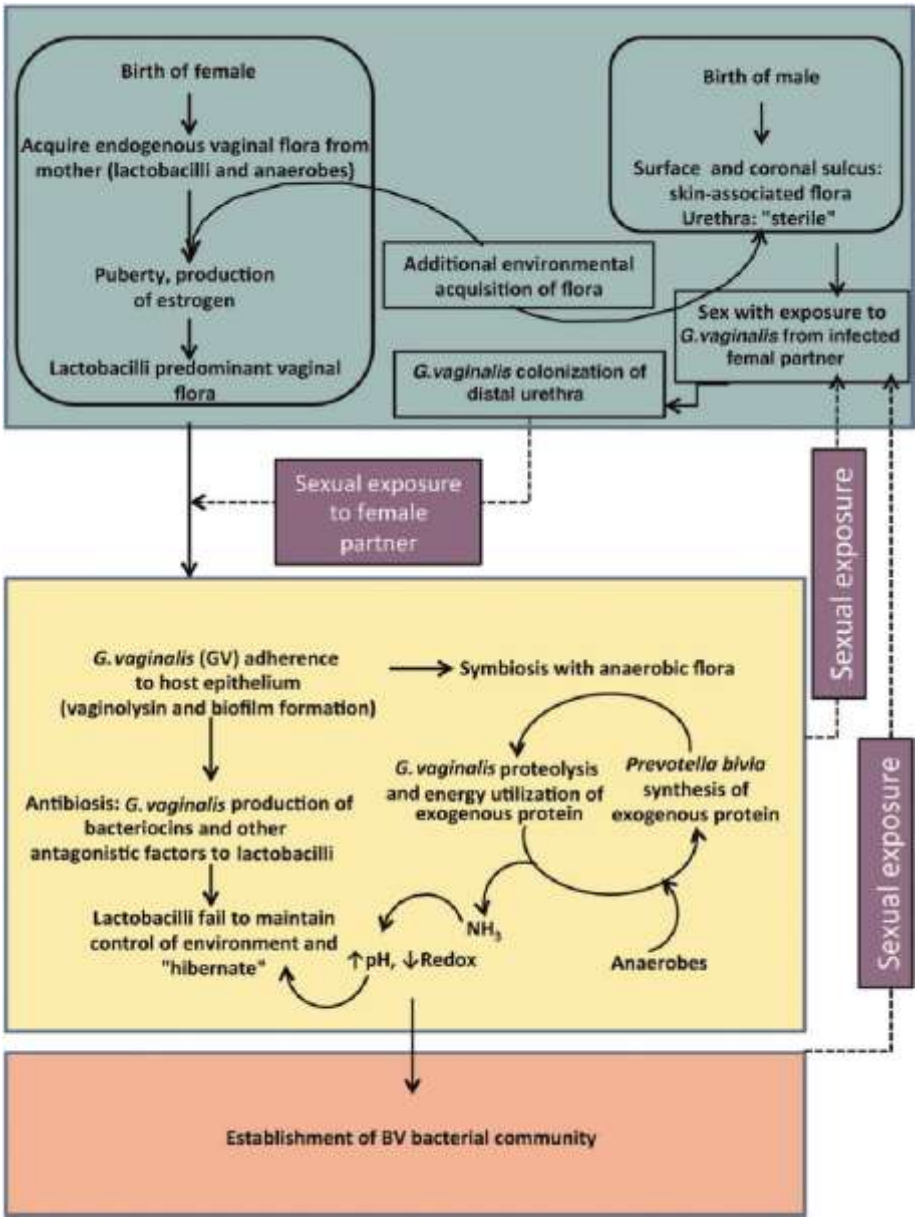


Figure 5: Transmission and pathogenesis of *Gardnerella vaginalis*. As depicted in the model, *G. vaginalis* does not constitute the normal vaginal flora at birth but is rather transmitted through sexual contact with an infected individual. Its ability to compete with normal vaginal flora for dominance is attributed to virulence factors that enable adherence to host vaginal epithelium. An elevated pH, and lowered reduction-oxidation (redox) potential resulting from *G. vaginalis* infection, enhances growth of host anaerobes and suppression of lactobacilli. Image taken from Schwebke et al., 2014 [100].

2.10. Genetic diversity of *G. vaginalis*

It is presumed that the difficulty of lysing *G. vaginalis* hindered early genetic explorations, therefore until 2010, virtually nothing was known about the genetics of *G. vaginalis* [12]. Early studies conducted using a variety of techniques indicated that the genome of *G. vaginalis* had a low (42–43.5 %) GC content [70, 71]. It was also suggested that the size of the *G. vaginalis* genome ranged between 1.67 Mb and 1.72 Mb in a study by Lim *et al.,* 1994 [101]. Studies employing genotyping of *G. vaginalis* later confirmed the genome of *G. vaginalis* to be 1.62–1.67 Mb with a low GC content (41–42%) [83].

Yeoman *et al.,* 2010 [83] reported great genomic diversity between different isolates, as well as differences in virulence potentials among several strains *G. vaginalis* following genome sequencing. Sequence analysis of the *G. vaginalis* 16S rRNA gene also showed that the bacterium is most closely related to *Bifidobacterium coryneforme* and *Bifidobacterium minimum* (Gram-positive organisms) [83]. Examination of virulence factors for a strain of *G. vaginalis* from a BV positive woman and another without BV reported impaired adherence in the non-BV isolate suggesting that there may be both commensal and pathogenic strains of *G. vaginalis* [78]. However, in accordance with other studies, there is no mention of the Amsel or Gram stain characteristics of the woman without BV. This suggests that this type of work needs to be replicated with multiple isolates. A study by Ahmed *et al.,* 2012 [102] agreed with the findings reported by Yeoman and colleagues as they also found that *G. vaginalis* was incredibly taxonomically diverse for a single species.

Recent comparative genomic analyses of 17 clinical isolates of *G. vaginalis* suggested that the species can be subdivided into 4 clades or even that there may be multiple species of *G. vaginalis* [102, 103]. In 2019, Vaneechoutte and colleagues [104] confirmed that *Gardnerella* did indeed consist of other species namely *G. leopoldii*, *G. piotti*, and *G. swidsinskii*. This

breakthrough was the result of whole-genome sequence analysis of 81 *Gardnerella* strains. The study reported the existence of at least 13 groups distinct enough to be classified as separate species, within the taxon formerly known as *G. vaginalis* [104].

2.11. Genotyping of *G. vaginalis*

Piot *et al.,* 1984 [105] found that *G. vaginalis* has been divided into at least 8 biotypes on the basis of its metabolic properties. A study by Ingianni *et al.,* 1997 [22] reported the use of several genotyping methods, particularly with deoxyribonucleic acid (DNA) restriction profiles as shown in Figure 6. However, due to great variability in genome fingerprinting of *G. vaginalis*, both restriction enzyme analysis (REA) and restriction fragment length polymorphism (RFLP) of the Southern blot of digests proved to be unsatisfactory for typing these species and for the definition of the isolated strains [106-108]. In addition, REA determined that more than one genotype of *G. vaginalis* may be present for BV patients, as well as a level of genomic heterogeneity amongst the *G. vaginalis* present in BV patients. It has also been claimed that a bacterial shift in a BV patient can take place over a period of time [106-108].

DNA fingerprinting-based analysis methods were shown to be ineffective with restriction profiles of *G. vaginalis*, therefore they would be unlikely to guarantee successful distinction between clinical isolates [108]. This finding was significant as it lead to the development of improved diagnostic methods [22, 109]. Genotypic and biotyping methods were developed to determine genotypic heterogeneity of *G. vaginalis* [22, 105], which proved useful in studying the taxonomy and epidemiology of *Gardnerella* [22]. However, these early typing assays often failed to reveal *G. vaginalis* diversity [14]. Since genotyping of *G. vaginalis* requires specificity, high-fidelity polymerase chain reaction (PCR) is the preferred diagnostic method since the DNA polymerase shows increased efficacy [110], resulting in increased accuracy in the replication of the DNA of interest.

The findings of Jayaprakash and colleagues [111] contributed significantly to diversity studies of *G. vaginalis* through sequencing of the gene encoding chaperonin 60 (cpn60). From the 112 *G. vaginalis* isolates, four subgroups (A–D) [112], further divided into four clades (1–4) [102] by whole-genome sequencing, were identified. The presence of the previously unknown subgroups may call for the four-group division to be expanded [112, 113].

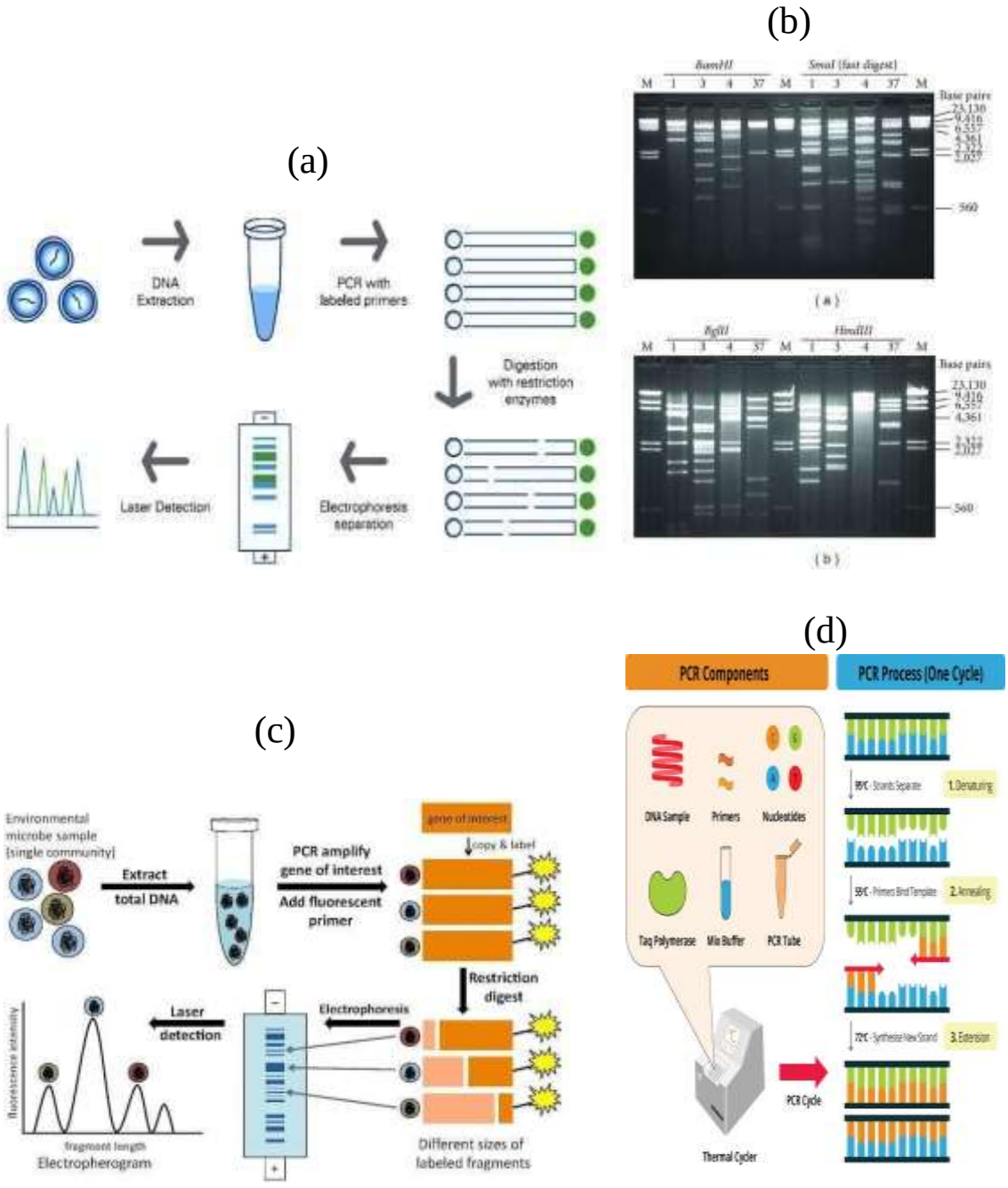


Figure 6: Methods employed in the genotyping of *Gardnerella vaginalis*. (a) ARDRA technique, (b) REA, (c) Terminal RFLP, and (d) PCR.

2.12. Amplified Ribosomal DNA Restriction Analysis

ARDRA is a genomic analysis tool, introduced in 1997, based on restriction endonuclease digestion of the amplified bacterial 16S rDNA (Figure 6a). It is a simple, rapid, reproducible method of genotyping/ ribotyping that results in DNA fingerprints obtained by using only one PCR reaction and one restriction enzyme (that can recognize DNA sequences as low as 4bp). It functions to discriminate among bacteria at genus levels by allowing for species identification, subdivision of bacterial strains into limited numbers of different genotypes, and strain characterisation in epidemiological investigations [114].

Ingianni and colleagues [22] found that the ARDRA method allowed for *G. vaginalis* to be separated into at least 4 genotypes, some of which showed a prevalent distribution in certain of the centers they were derived from. It has also been demonstrated by other authors to be very useful for microbial molecular epidemiology and taxonomy [20]. In ARDRA, bacterial *rRNA* genes are first amplified by PCR using conserved sequences of DNA as primers. The positive PCR amplicons then undergo restriction endonuclease digestion followed by the resolve of restriction fragments electrophoretically to obtain a fingerprint. ARDRA fingerprinting is advantageous as it is faster to perform than classic ribotyping [114], and is less error prone [22, 21]. The results of this molecular diagnostic tool assists in understanding the ecology and clinical significance of a wide range of bacterial pathogens [20] and the roles they play in the spread and persistence of infection [22].

The environmental applications of ARDRA ensures: rapid monitoring of microbial communities in environmental samples, comparison of microbial diversity in response to altered environmental conditions, characterisation of microbial communities during the biodegradation process, and the identification of unique clones and estimation of the operational taxonomic units in environmental clone libraries based on the restriction profiles

of clones [115]. However, with environmental samples, the major limitation encountered in ARDRA analysis is that the restriction profiles generated from complex microbial communities are sometimes too difficult to be resolved by agarose or polyacrylamide gel electrophoresis [116].

With clinical samples, ARDRA was determined to be a superior molecular diagnostic tool when compared to REA and RFLP due to its ability to discriminate *G. vaginalis* into homogenous subtypes [22]. In addition, the study by Pleckaityte *et al.*, 2012 [23], showed that the use of ARDRA in establishing the link between *G. vaginalis* isolates and sialidase production could be considered a possible marker for the identification of pathogenic potential of *G. vaginalis* strains.

Although ARDRA is an advantageous genotypic method, it has been shown to produce variable results in terms of *G. vaginalis* differentiation, with limited success at consistently implicating a particular biotype with BV [23, 111]. Associations between genotypic and phenotypic characteristics, or between genotype/phenotype with demographic or clinical characteristics [23, 111, 117], have hardly been successfully described.

CHAPTER 3

MATERIALS & METHODS

3.1. Ethics approval

The study was approved by the Biomedical Research Ethics Committee (BREC) of UKZN (BREC/00000093/2019), (Appendix 1).

3.2. Study design and setting

This study was a sub-study of a larger study which focused on laboratory-based detection of vaginitis pathogens in pregnant women. The larger study included women, 18 years and older who were willing to provide written informed consent and willing to be tested for vaginal pathogens. The study population was recruited from October 2017 to April 2018. The enrolled women (n=354) provided self-collected vaginal swabs after receiving instructions from the study staff on the method of sample collection. Samples were obtained from women from gestational age 12 weeks to 37 weeks. At enrolment, all women also provided data on their socio-demographic statuses (age, marital status, level education), sexual behaviour (age of first sex, cohabitation status, condom use and number life time sexual partners) and clinical history (trimester of pregnancy, history of STIs, previous pregnancy history). The data was collected using a structure questionnaire.

The women were classified as BV negative, intermediate and positive using the Nugent scoring criteria on gram-stained vaginal smears. A 100% in-house quality control check on the gram-stained slides was performed. The study was conducted at the School of Clinical Medicine Research Laboratory at the Nelson R. Mandela School of Medicine, UKZN.

3.3. Laboratory procedures

3.3.1. Nugent scoring for grading of vaginal smears

Smears were prepared from vaginal swabs and rolled onto glass slides. The slides were gram stained and examined under the oil immersion objective. Each slide was then graded as per the standardised quantitative morphological classification method developed by Nugent *et al*., 1991 [64], which has been described in the literature review.

3.3.2. DNA extraction

DNA was extracted from the vaginal swabs using a commercially available kit, Purelink Microbiome DNA purification kit (ThermoFisher Scientific, Massachusetts, United States) according to manufacturer's instructions.

For preparation of the lysate, the sample was centrifuged at 14,000 × *g* for 10 minutes to pellet the microorganisms. The supernatant was carefully removed and discarded. Thereafter, the microbial pellet was re-suspended in 800µl of S1- Lysis Buffer, pipetted up and down to re-suspend, and the sample was transferred to the Bead Tube. Thereafter, 100µl of S2- Lysis Enhancer was added, capped securely, and vortexed briefly. The sample was incubated 95°C for 10 minutes. Following incubation, bead beating for 10 minutes at maximum speed on a vortex mixture was carried out to homogenise the sample. The sample was then centrifuged at 14,000 × *g* for 10 minutes. Following centrifugation, approximately 500µl of the supernatant was transferred to a clean microcentrifuge tube.

This was followed by the addition of 900µl of S4- Binding Buffer to the supernatant and brief vortexing. After mixing, 700µl of the sample mixture was loaded onto a spin column-tube assembly, and centrifuged at 14,000 × *g* for 1 minute. The flow-through was discarded, and the previous step, repeated, with the remaining sample mixture.

The final wash and elution step of the DNA extraction process included placing the spin column in a clean collection tube with the addition of 500µl of S5- Wash Buffer, and centrifugation at 14,000 × *g* for 1 minute. The flow-through was discarded, followed by centrifugation of the spin column-tube assembly at 14,000 × *g* for 30 seconds. The spin column was placed in a clean tube and 100µl of S6- Elution Buffer was added. An incubation step followed at room temperature for 1 minute. Finally, the spin column-tube assembly was centrifuged at 14,000 × *g* for 1 minute, and the column was discarded.

3.3.3. PCR amplification of the 16S rRNA genes of *G. vaginalis*

The 16S rRNA gene specific to *G. vaginalis* was amplified using primers: Forward: 5′-TTCGATTCTGGCTCAGG and Reverse: 5′-CCATCCC AAAAGGGTTAGGC. The primers were synthesised based on their published sequences described by Pleckaityte *et al.*, 2012 [23]. The PCR was performed in a 50µL final volume and comprised [1µl] of each primer, [4µl] of genomic DNA and [25µl] of High Fidelity PCR enzyme mix (ThermoFisher Scientific, Massachusetts, United States). The reaction mixture was subjected to 28 cycles of denaturation at 94°C for 30 seconds, primer annealing at 52°C for 45 seconds and extension at 72°C for 1 minute 25 seconds.

PCR conditions were as per Pleckaityte *et al.*, 2012 [23]. All PCR reactions were performed using a T100 thermocycler (BioRad, California, United States). The PCR products were separated on a 1% agarose gel and viewed under a UV transilluminator (Gene Genius, SYNGENE).

3.3.4. Sequencing of 16S rRNA gene PCR amplicons

To confirm the identity of the PCR amplicons prior to genotyping, the amplicons were sequenced using the Sanger method [118] at Inqaba Biotechnological Industries in Pretoria, South Africa. The amplicons were sequenced using an ABI3500XL genetic analyser and the raw sequence data was edited using Chromas software V2.6.5 (Technelysium, Queensland, Australia). The edited forward and reverse sequences were aligned using the DNAMAN software (Lynnon Biosoft, California, United States) and the identity of the edited sequences was confirmed using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST).

3.3.5. ARDRA

Positive 16S rRNA gene PCR amplicons were subjected to restriction digestions with the *Taq*I enzyme (Thermo Scientific, South Africa) at 65°C for 3 hours. Resulting restriction digested products were analyzed on a 1.5% agarose gel (TopVision LE GQ Agarose, Thermo Scientific, South Africa) and visualised using a UV transilluminator (Gene Genius System). The positive amplicons were also digested with *BamH*I and *Hind*III restriction enzymes (Thermo Scientific, South Africa) in order to determine the genotypic subtypes that are present. The digests were incubated at 37°C for 4 hours, followed by a heat inactivation step at 65°C for 15 minutes. Restriction fragments were analyzed by agarose gel electrophoresis on a 1.5% gel (TopVision LE GQ Agarose, Thermo Scientific, South Africa). Assignment of subtypes were performed by correlating the *BamH*I and *Hind*III restriction profiles according to Pleckaityte *et al*., 2012 [18].

3.3.6. Phylogenetic analysis

A phylogenetic tree was then constructed from the 16S rRNA gene sequence data using the Molecular Evolutionary Genetics Analysis (MEGA) version 10 software (Arizona, United States). A bootstrap consensus tree inferred from 1000 replicates using the Neighbour-Joining method was generated.

3.3.7. Data analyses

The data analysis was performed in R Statistical Computing software, version 3.6.2. To assess the association between the symptoms and the BV status for each genotype, the Chi-Square goodness of fit test for one sample was used. The results were also presented as component bar charts.

CHAPTER 4

RESULTS

4.1. Diagnosis of BV in the study population

Of the 354 samples analysed, 124 were BV positive, 37 were BV intermediate and 193 were

BV negative. The remaining slides (100) were unreadable due to poor quality of the slide (inadequate sample material on slide). We randomly selected 50 BV negative, 37 BV intermediate and 50 BV positive specimens for *G. vaginalis* detection and genotypic analysis. A total of 137 samples were analysed.

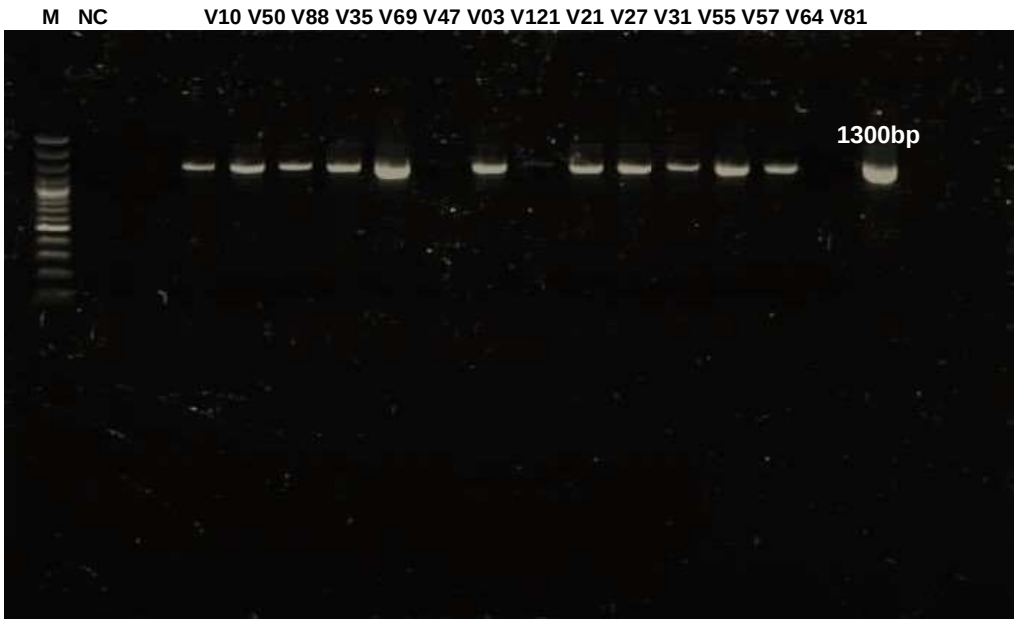
4.2. PCR amplification of the 16S rRNA genes *of G. vaginalis*

A 1300bp fragment corresponding to the 16S rRNA gene of *G. vaginalis* was amplified in 37/137 (27.2%) study samples analysed (Figure 7). The 16S rRNA gene was not detected in any of the BV negative samples (0/50). A BV negative group was therefore not included in any further analysis. From the 50 BV positive samples based on Nugent scoring, only 23 samples produced the 1300bp product. In addition, 14/37 BV intermediate samples tested positive for GV (Figure 8). Attempts to detect GV from the unsuccessful samples were made such as increasing the concentration of template DNA and adjusting primer and amplification conditions. All attempts were unsuccessful.

The possibility of sample inhibitors affecting the PCR reactions or failed DNA extractions were ruled out since the same DNA samples were amplifiable for other genes not included in

this study. A set of 37 samples (23 BV positives and 14 BV intermediates) were used for further analysis.

a)



b)

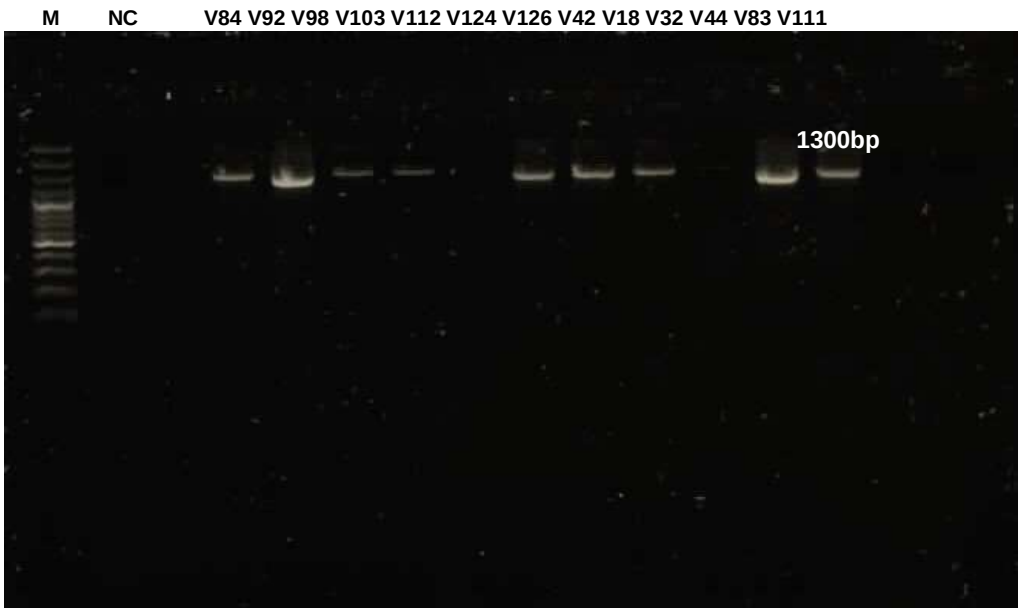


Figure 7: *Gardnerella vaginalis* polymerase chain reaction results from bacterial vaginosis positive samples by agarose gel electrophoresis. Lane M: 100bp molecular weight marker (ThermoFisher Scientific), Lane NC: Negative control (no template DNA), and Lanes 1-15 (a); and 1-13 (b): BV positive samples. Only 23 BV positive samples produced the 1300bp product.

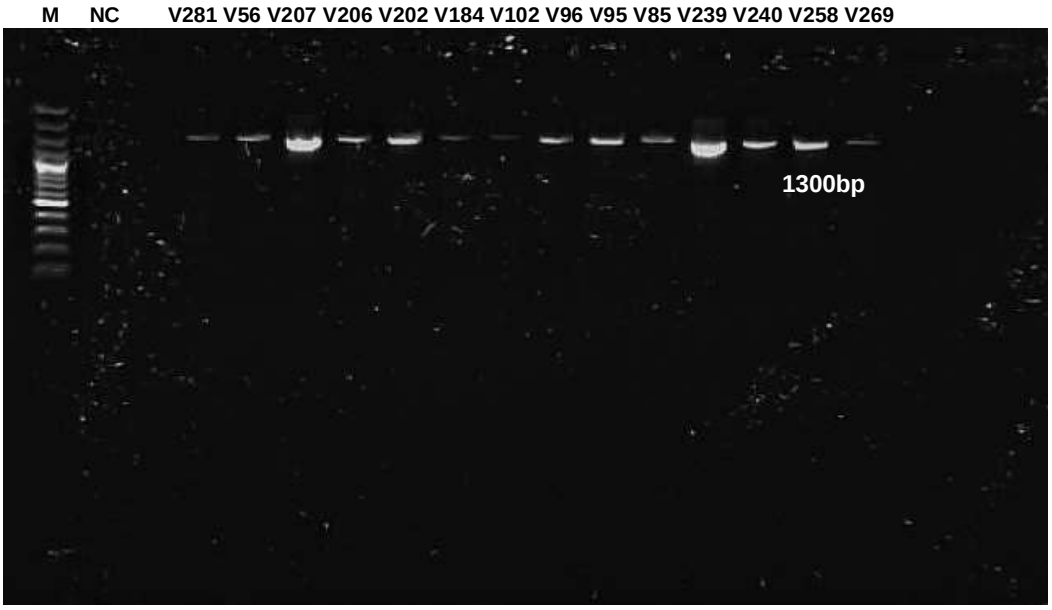


Figure 8: Gardnerella vaginalis polymerase chain reaction results from bacterial vaginosis intermediate samples by agarose gel electrophoresis. Lane M: 100bp molecular weight marker (ThermoFisher Scientific), Lane NC: Negative control (no template DNA), could not amplify thereby validating the experiment, and Lanes 1-14: BV intermediate samples.

4.3. Sequencing of 16S rRNA *gene* PCR amplicons

All samples were successfully sequenced. The majority of the samples produced the same BLAST hit. A subset of the sequencing hits are shown in Table 1. The DNA sequencing hits of the 16S rRNA gene showed identity (97%) to *G. vaginalis* strain GS10234 (MH898659.1) and *G. vaginalis* strain N153 (98%) (JQ354973.1) (Table 1).

Table 1: Basic Local Alignment Search tool (BLAST) results for 16S rRNA gene amplicons of *Gardnerella vaginalis*

Query sequence	BLAST hit	Percentage identity (%)	Accession number
V18	*Gardnerella vaginalis* strain GS10234 16S ribosomal RNA gene	97.17%	MH898659.1
V88	*Gardnerella vaginalis* strain GS10234 16S ribosomal RNA gene	97.86%	MH898659.1
V92	*Gardnerella vaginalis* strain GS10234 16S ribosomal RNA gene	98.60%	MH898659.1
V96	*Gardnerella vaginalis* strain N153 16S ribosomal RNA gene	98.45%	JQ354973.1
V102	*Gardnerella vaginalis* strain GS10234 16S ribosomal RNA gene	97.33%	MH898659.1
V207	*Gardnerella vaginalis* strain GS10234 16S ribosomal RNA gene	98.45%	MH898659.1

4.4. Genotyping analysis

The distribution of the genotypes based on *Taq*I digestion for the 37 specimens analysed is shown in Table 2. The subtypes of the genotypes which were determined by combining the banding profiles of *BamH*I and *Hind*III digests are also presented in Table 2.

4.4.1. Genotypes based on *Taq*I digestion

Restriction digestion with *Taq*I revealed the presence of two different genotypes i.e. GT1 and GT2. GT1 was carried by 20/37 specimens (54%), followed by GT2 which was present in 9/37 specimens (24%). Of the 37 specimens analysed, 7 specimens were not ascribed genotypes. Two of the specimens from the BV positive sample group produced a banding profile (i.e. a single band at 500bp) that was not described in previous published studies. One specimen from the BV intermediate group produced a very faint profile which was difficult to interpret. The

remaining 3 specimens did not produce any bands, the gel lanes appeared blank for those samples. These specimens were across both BV status groups.

Within the BV positive sample group, 13/23 specimens carried GT1 (56.5%) and 6 of the 23 specimens (26.1%) carried GT2. Two specimens produced a differing banding profile (8.7%) and 2 specimens did not produce any bands (8.7%) (Figure 9).

A similar profile was observed for the BV intermediate sample group, a larger number of specimens carried GT1 (7/14, 50.0%) and 3 out of 14 samples carried GT2 (21.4%). Three samples did not produce visible bands (21.4%) (Figure 10).

4.4.2. Subtypes based on *BamH*I and *Hind*III digests

Within the BV positive sample group, all 4 subtypes were observed. Subtype 2B was highly prevalent with 11/23 (47.8%) specimens harbouring this subtype followed by the mixed 2AB subtype (4/23, 17.4%), subtype C (3/23, 13.0%) and subtype 1 (2/23, 8.7%) (Figure 11). In the sample group that carried GT1 (n=13), 5 specimens' harboured subtype 2B (38.5%), 3 with subtype 2C (23.1%), 2 with subtype 2AB (15.3%) and 2 with subtype 1 (15.3%) (Table 2). Subtype 2B was highly prevalent in the sample group carrying GT2 (5/6, 83.3%), followed by subtype 2AB (1/6, 16.6%). Subtypes 1 and C were not present in this genotypic group (Table 2).

Within the BV intermediate sample group, 3 subtypes were observed (Subtypes, 1, 2B and C) (Figure 12). The most prevalent subtype in this group was subtype 1 (9/14, 64.2%) followed by subtype 2B (4/9, 44.4%) and subtype 2C (1/9, 11.1%). Subtypes 1 (3/7, 42.8%) and 2B (3/7, 42.8%) were most prevalent in GT1 specimens. One specimen in this genotypic group carried subtype 2C (7.14%) (Table 2). Subtype 1 was also shown to be most prevalent in the GT2

specimens (2/3, 66.7%) followed by subtype 2B (1/3, 33.3%). Subtype 2C was not present in this genotypic group (Table 2).

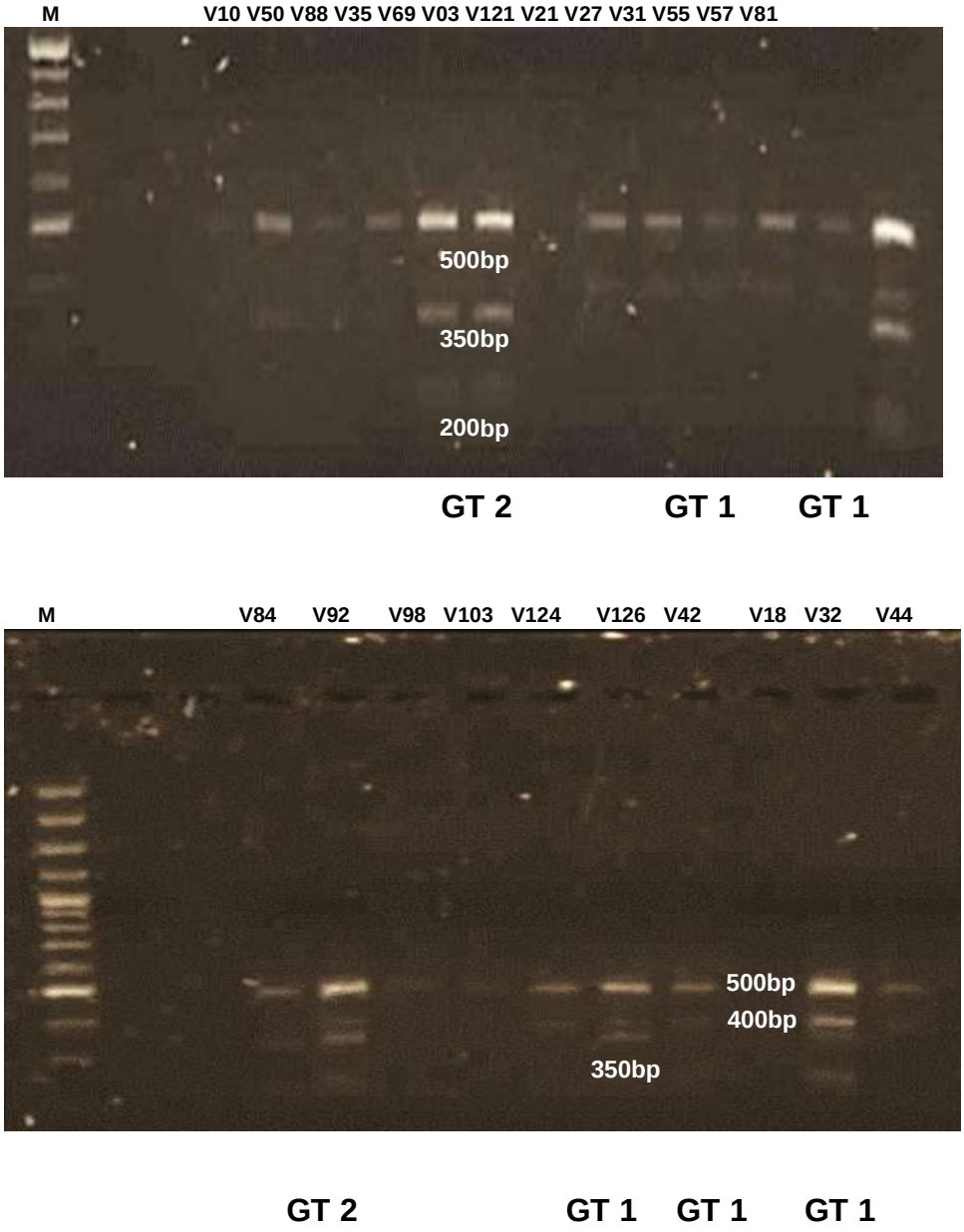


Figure 9: Profiles of *Gardnerella vaginalis* detected in bacterial vaginosis positive samples digested with *Taq*I. Lanes M: 100bp DNA molecular ladder (ThermoFisher Scientific). Genotypes 1 (100bp, 350bp, 400bp, 500bp banding pattern) and 2 (100bp, 200bp, 350bp, 500bp banding pattern) were distributed across

BV positive samples.

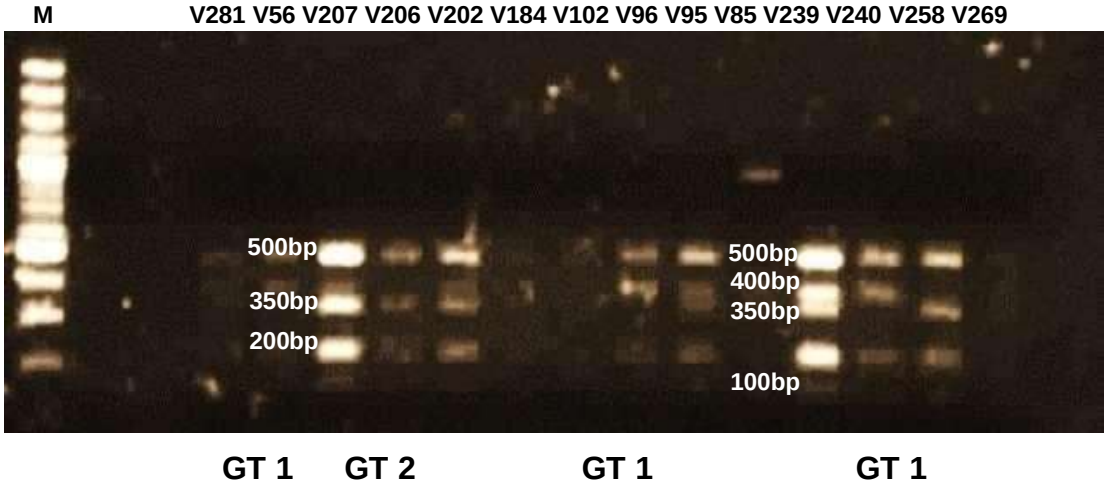
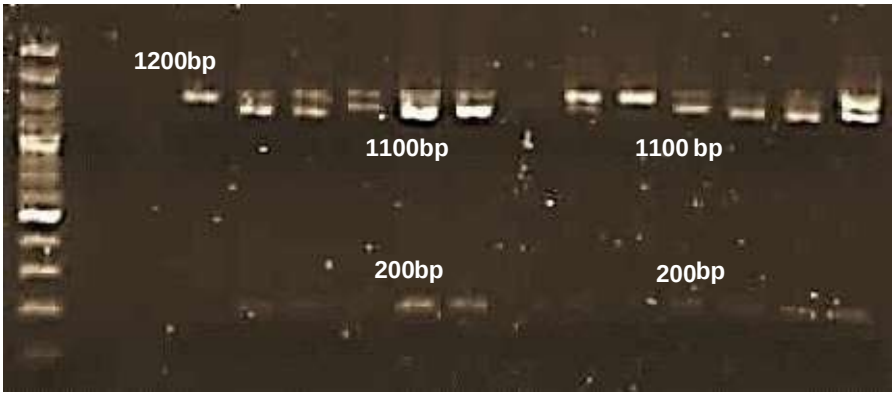
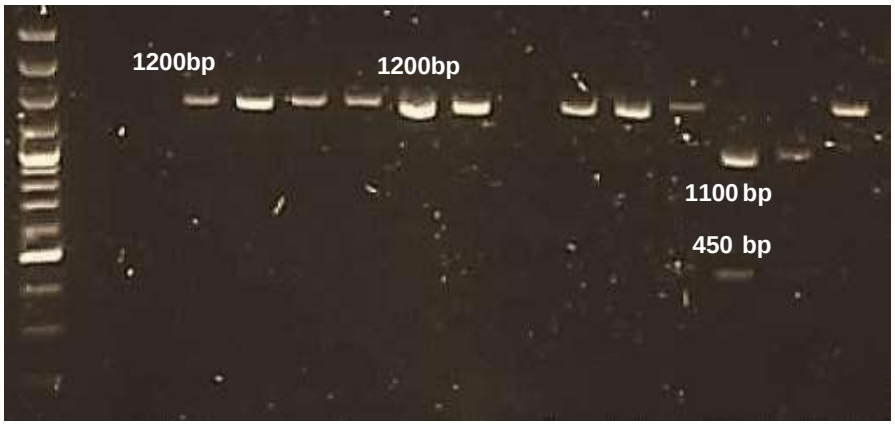


Figure 10: Profiles of *Gardnerella vaginalis* detected in bacterial vaginosis intermediate samples digested with *Taq*I. Lane M: 100bp DNA molecular ladder (ThermoFisher Scientific). Genotypes 1 (100bp, 350bp, 400bp, 500bp banding pattern) and 2 (100bp, 200bp, 350bp, 500bp banding pattern) were distributed across BV intermediate samples.

M V10 V50 V88 V35 V69 V03 V121 V21 V27 V31 V55 V57 V81



1 **2B** **2C**



M V10 V50 V88 V35 V69 V03 V121 V21 V27 V31 V55 V57 V81

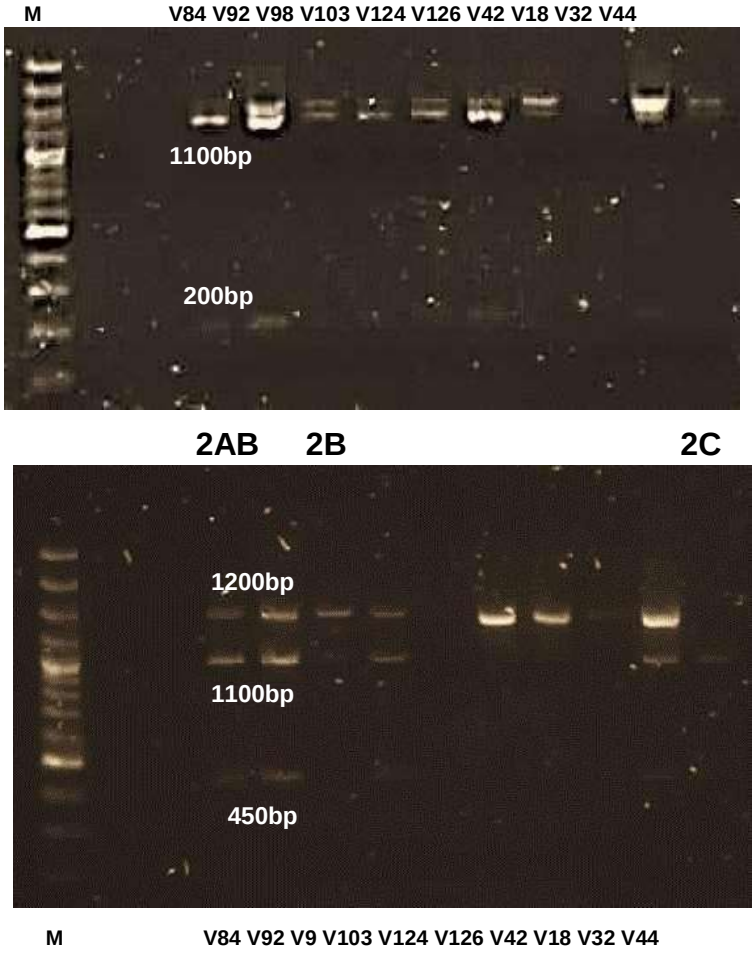


Figure 11: Subtypes assigned to *Gardnerella vaginalis* detected in bacterial vaginosis positive samples based on *BamH*I and *Hind*III digestion profiles. Lane M: 100bp DNA molecular ladder (ThermoFisher Scientific). Subtypes 1 (1200bp band for *BamH*I and *Hind*III digestion), 2B (200bp, 1100bp for *BamH*I digestion and

1200bp for *Hind*III digestion), 2AB (200bp, 1100bp for *BamH*I digestion and 450bp, 1000bp, 1200bp for *Hind*III digestion) and 2C (200bp, 1100bp for *BamH*I digestion and 450bp, 1000bp for *Hind*III digestion) were distributed across the BV positive samples.

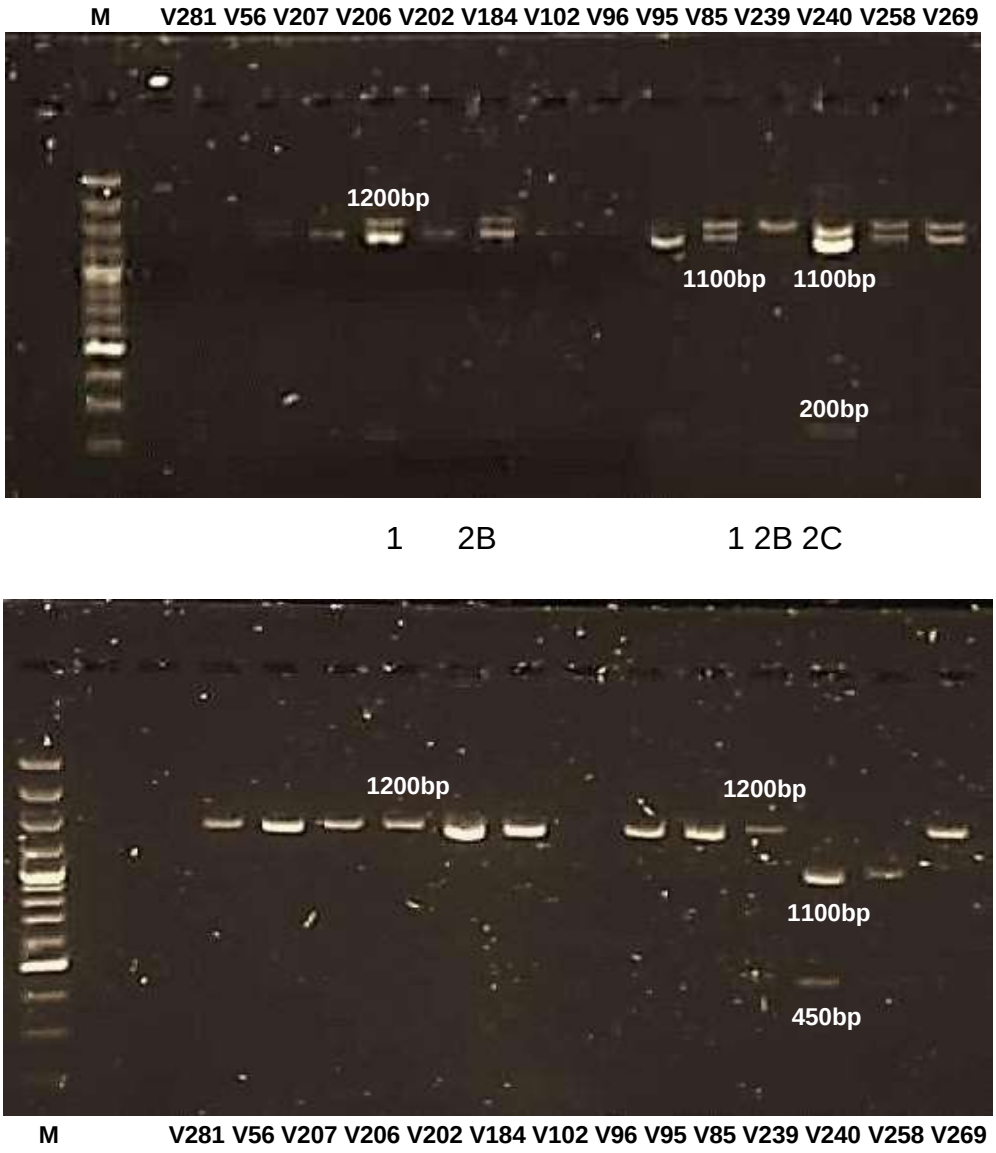


Figure 12: Subtypes assigned to *Gardnerella vaginalis* detected in bacterial vaginosis intermediate samples based on *BamH*I and *Hind*III digestion profiles. Lane M: 100bp DNA molecular ladder (ThermoFisher Scientific). Subtypes 1 (1200bp band for *BamH*I and *Hind*III digestion), 2B (200bp, 1100bp for *BamH*I digestion and 1200bp for *Hind*III digestion) and 2C (200bp, 1100bp for *BamH*I digestion and 450bp, 1000bp for *Hind*III digestion) were distributed across the BV intermediate samples.

Table 2: Genotypes identified after digestion with *Taq*I across bacterial vaginosis intermediate and bacterial vaginosis positive women. Subtypes identified after digestion with *BamH*I and *Hind*III

Sample Name	*Taq*I fragment sizes	Genotype	*BamH*I fragments sizes	*Hind*III fragment sizes	*Subtype*
BV positives					
V003	100bp, 200bp, 350bp, 500bp	2	200bp, 1100bp	1200bp	2B
V010	350bp, 400bp	1	1200bp	1200bp	1
V018	Bands not visible	-	Bands not visible	Bands not visible	-
V021	100bp, 350bp, 400bp, 500bp	1	200bp, 1100bp	1200bp	2B
V027	100bp, 350bp, 400bp, 500bp	1	1200bp	1200bp	1
V031	100bp, 350bp, 400bp, 500bp	1	200bp, 1100bp	1200bp	2B
V032	250bp, 350bp, 400bp, 500bp	1	200bp, 1100bp	450bp, 1000bp, 1200bp	2AB
V035	350bp, 500bp	2	200bp, 1100bp	1200bp	2B
V042	400bp, 500bp	1	200bp, 1100bp	1200bp	2B
V044	400bp, 500bp	1	200bp, 1100bp	450bp, 1000bp	2C
V050	100bp, 200bp, 350bp, 500bp	2	200bp, 1100bp	1200bp	2B

V055	100bp, 350bp, 400bp, 500bp	1	200bp, 1100bp	450bp, 1000bp	2C
V057	100bp, 350bp, 400bp, 500bp	1	200bp, 1100bp	450bp, 1000bp	2C
V069	100bp, 200bp, 350bp, 500bp	2	200bp, 1100bp	1200bp	2B
V081	100bp, 350bp, 400bp, 500bp	1	200bp, 1100bp	1200bp	2B
V084	100bp, 200bp, 350bp, 500bp	2	200bp, 1100bp	450bp, 1000bp, 1200bp	2AB
V088	350bp, 500bp	2	200bp, 1100bp	1200bp	2B
V092	100bp, 350bp, 400bp, 500bp	1	200bp, 1100bp	450bp, 1000bp, 1200bp	2AB
V098	500bp	Different pattern	200bp, 1100bp	1200bp	2B
V103	500bp	Different pattern	200bp, 1100bp	450bp, 1000bp 1200bp	2AB
V121	Bands not visible	-	Bands not visible	Bands not visible	-
V124	400bp, 500bp	1	200bp, 1100bp	Bands not visible	-
V126	100bp, 350bp, 400bp, 500bp	1	200bp, 1100bp	1200bp	2B
BV intermediates					
V056	250bp, 400bp, 500bp	1	1200bp	1200bp	1
V085	Undigested DNA	-	1200bp	1200bp	1
V095	250bp, 350bp, 400bp, 500bp	1	200bp, 1100bp	1200bp	1

V096	250bp, 400bp, 500bp	1	200bp, 1100bp	1200bp	2B
V102	Very faint pattern	-	1200bp	1200bp	1
V184	No band visible	-	1200bp	1200bp	1
V202	250bp, 350bp, 500bp	2	200bp, 1100bp	1200bp	1
V206	250bp, 350bp, 500bp	2	1200bp	1200bp	1
V207	250bp, 350bp, 500bp	2	200bp, 1100bp	1200bp	2B
V239	250bp, 350bp, 400bp, 500bp	1	200bp, 1100bp	400bp, 1000bp	2B
V240	250bp, 400bp, 500bp	1	200bp, 1100bp	1200bp	2B
V258	250bp, 400bp, 500bp	1	200bp, 1100bp	450bp, 1000bp	2C
V269	No bands visible	-	1200bp	1200bp	1
V281	250bp, 400bp, 500bp	1	1200bp	1200bp	1

4.5. Phylogenetic analysis of 16S rRNA gene genotypes and subtypes

The phylogenetic tree revealed the presence of 5 clusters (Figure 13). The tree displayed clusters which contained groups of specimens from a particular BV status (clusters 1, 3 and 5). Within these same status groups there were however differences noted for either the genotypes assigned and/or subtypes present. Additionally, there were clusters which contained specimens from across both BV status groups such as cluster 2 and 4. Despite the heterogeneity with respect to BV group, cluster 2 contained specimens of the same genotype (GT1) with the majority carrying the same subtype (S2B). Cluster 4 on the other hand, contained specimens of the same genotype (GT1) with a combination of all 4 subtypes (S1, S2B, S2AB, and S2C).

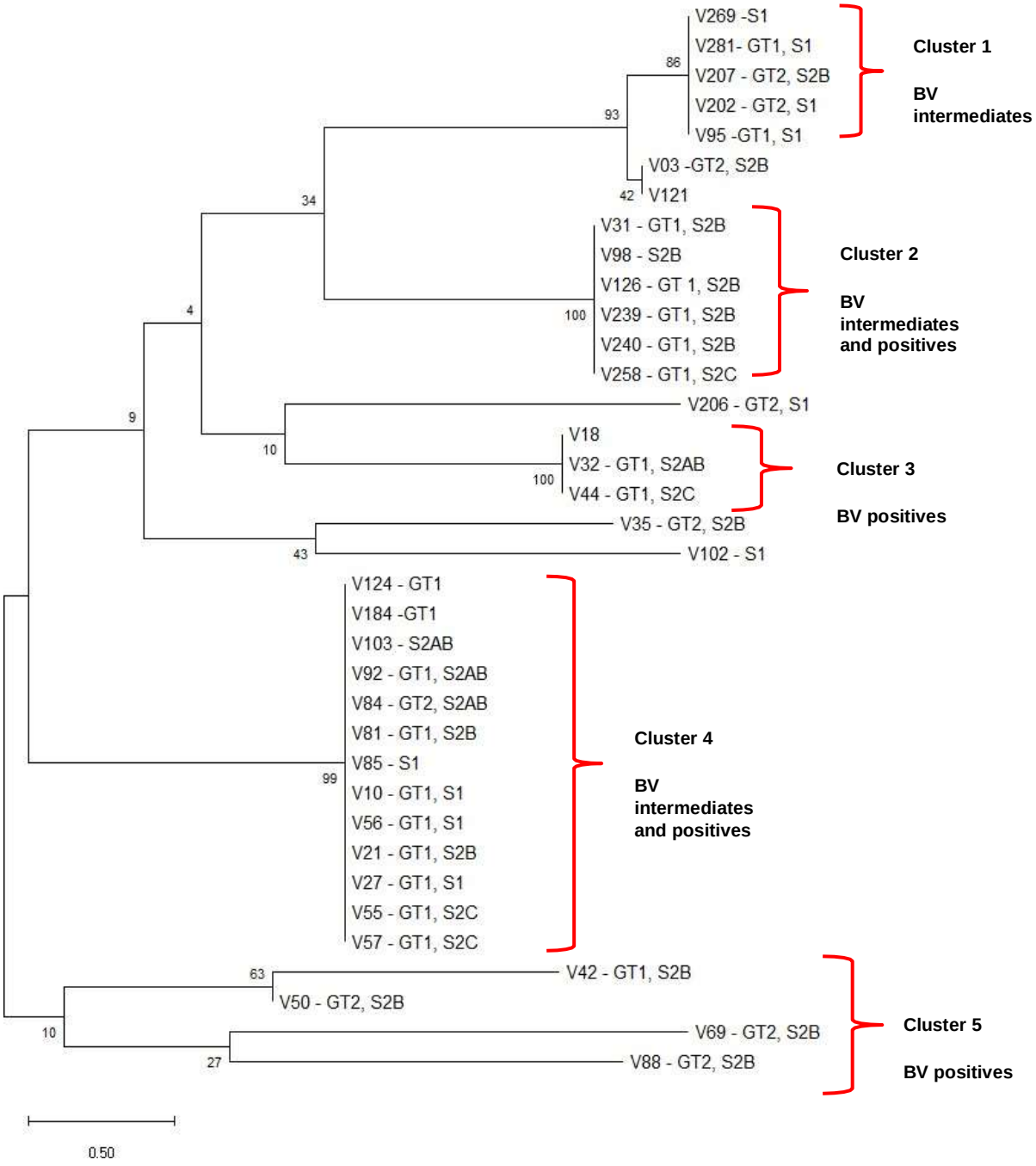


Figure 13: Phylogenetic analysis according to distribution of genotypes. The tree was constructed using the Neighbour-Joining method. The optimal tree with the sum of branch length = 15.35302543 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using

the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA X.

4.6. Characteristics of the genotyped study population

The characteristics of the study population is described in Table 3. Within the overall study population, 62.2% did not experience current abnormal vaginal discharge compared to the 37.8% that did. A higher proportion (64.9%) of the study women attended high school. A lower proportion (27%) of these women attended college/ university, whilst 8.1% completed primary school only. Majority of the women in the overall study population were unmarried (94.6%), had a regular sex partner (83.8%), did not co-habit with their sex partner (56.8%), had sex for the first time between the ages of 15 and 20 (81.8%), had 2-4 lifetime sex partners (45.9%), said their partner has other partners (40.5%), used condoms only "sometimes" (59.5%), did not use a condom during their last sexual act (73%), did not smoke (91.9%), did not consume alcohol (89.2%), did not use intravaginal practices (94.6%), reported having past abnormal vaginal discharge (51.4%) , and were in the third trimester of pregnancy (48.6%). In addition, majority of the women did not experience having past preterm delivery (81.1%), past miscarriage (78.4%), past abortion (94.6%), or past treatment for STIs (62.6%) (Table 3).

When comparing the variables across the BV intermediate and positive groups, it was observed that across the both groups the majority of the women did not report symptoms of abnormal vaginal discharge when enrolled into the study. There was no significant association between presence of discharge and BV status (p=0.234). With respect to level of education, for both BV intermediate and positive women, a higher proportion had attended high school, however this was not significant (64.3% and 65.2%, p=0.383). There was also no significant association between marital status and BV status (p=0.517). For both BV intermediate and positive women, the majority of the women were unmarried (100% and 91.3%). There was also no significant

association (p>0.05) between co-habitation status, age of first sex, lifetime number of sex partners, partner having other partners, condom use at last sex act, smoking, consuming alcohol, engaging in intravaginal practices, trimester of pregnancy, previous pregnancy history and treatment for past STIs in relation to BV status (Table 3). There was a significant association between BV positive status and having a regular sex partner (p=0.021). A higher proportion of BV positive women reported having a regular sex partner when compared to the BV intermediate women (95.7% versus 64.3%). However, there were also significant associations between practicing condom use and experiencing past abnormal vaginal discharge in relation to BV status. With respect to using condoms, a higher proportion of BV positive women reported never using condoms when compared to the BV intermediate group (43.5% versus 7.1%, p=0.009). A higher proportion of women who reported experiencing past abnormal vaginal were in the BV intermediate group when compared to the BV positive group (71.4% versus 39.1%, p=0.057) (Table 3).

Table 3: Characteristics of the genotyped study population according to bacterial vaginosis status

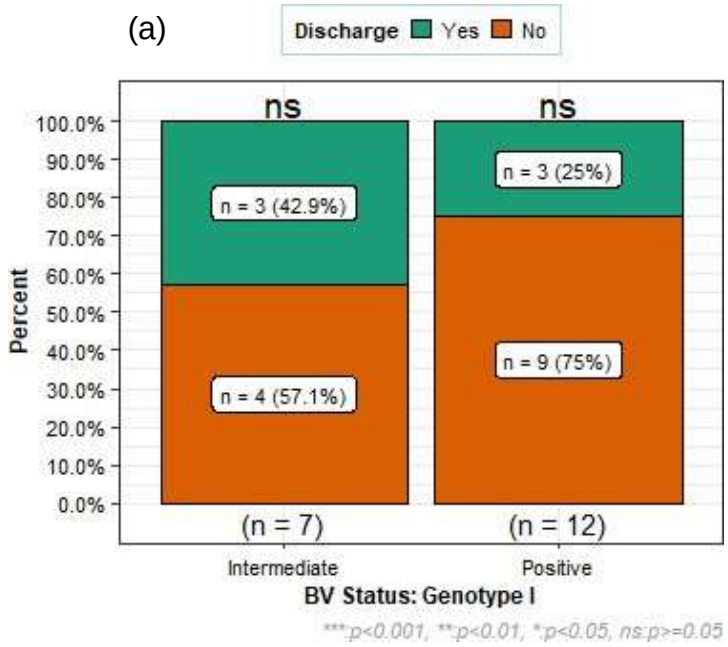
	BV status			
Variable	**Intermediate (n=14)**	**Positive (n=23)**	**p-value**	**Overall (n=37)**
Age			0.182	
Mean±SD(CV%)	25.6±4.47(17.5)	27.9±5.26(18.9)		27.0±5.04(18.7)
Median(Q1-Q3)	26.5(21.3-28.8)	26.0(24.0-30.5)		26.0(24.0-30.0)
Min-Max	19.0-33.0	18.0-38.0		18.0-38.0
Current abnormal vaginal discharge			0.234	
No	7 (50.0%)	16 (69.6%)		23 (62.2%)
Yes	7 (50.0%)	7 (30.4%)		14 (37.8%)

Level of education			0.383	
College/University	5 (35.7%)	5 (21.7%)		10 (27.0%)
High school	9 (64.3%)	15 (65.2%)		24 (64.9%)
Primary school	0 (0%)	3 (13.0%)		3 (8.1%)
Married			0.517	
No	14 (100%)	21 (91.3%)		35 (94.6%)
Yes	0 (0%)	2 (8.7%)		2 (5.4%)
Has a regular sex partner			0.021	
No	5 (35.7%)	1 (4.3%)		6 (16.2%)
Yes	9 (64.3%)	22 (95.7%)		31 (83.8%)
Living with sex partner			0.970	
No	8 (57.1%)	13 (56.5%)		21 (56.8%)
Yes	6 (42.9%)	10 (43.5%)		16 (43.2%)
Age of first sex			0.830	
<15	1 (7.1%)	2 (8.7%)		3 (8.1%)
15-20	11 (78.6%)	19 (82.6%)		30 (81.1%)
21-25	2 (14.3%)	2 (8.7%)		4 (10.8%)
Lifetime number of sex partners			0.222	
>4	3 (21.4%)	4 (17.4%)		7 (18.9%)
1	7 (50.0%)	6 (26.1%)		13 (35.1%)
2-4	4 (28.6%)	13 (56.5%)		17 (45.9%)
Partner has other partners			1.000	
Don’t know	5 (35.7%)	9 (39.1%)		14 (37.8%)
No	3 (21.4%)	5 (21.7%)		8 (21.6%)
Yes	6 (42.9%)	9 (39.1%)		15 (40.5%)
Practices condom use			0.009	
Always	1 (7.1%)	0 (0%)		1 (2.7%)
Never	1 (7.1%)	10 (43.5%)		11 (29.7%)
Rarely	0 (0%)	3 (13.0%)		3 (8.1%)
Sometimes	12 (85.7%)	10 (43.5%)		22 (59.5%)
Used a condom at last sex act			1.000	
No	10 (71.4%)	17 (73.9%)		27 (73.0%)
Yes	4 (28.6%)	6 (26.1%)		10 (27.0%)
Smokes			0.275	
No	14 (100%)	20 (87.0%)		34 (91.9%)
Yes	0 (0%)	3 (13.0%)		3 (8.1%)
Consumes alcohol			0.276	
No	14 (100%)	19 (82.6%)		33 (89.2%)
Yes	0 (0%)	4 (17.4%)		4 (10.8%)
Engages in intravaginal practices			1.000	
No	13 (92.9%)	22 (95.7%)		35 (94.6%)
Yes	1 (7.1%)	1 (4.3%)		2 (5.4%)
Trimester of pregnancy			0.605	

1st	3 (21.4%)	4 (17.4%)		7 (18.9%)
2nd	3 (21.4%)	9 (39.1%)		12 (32.4%)
3rd	8 (57.1%)	10 (43.5%)		18 (48.6%)
Had a past pre-term baby			1.000	
No	11 (78.6%)	19 (82.6%)		30 (81.1%)
Yes	3 (21.4%)	4 (17.4%)		7 (18.9%)
Had a past miscarriage			0.683	
No	12 (85.7%)	17 (73.9%)		29 (78.4%)
Yes	2 (14.3%)	6 (26.1%)		8 (21.6%)
Had a past abortion			1.000	
No	13 (92.9%)	22 (95.7%)		35 (94.6%)
Yes	1 (7.1%)	1 (4.3%)		2 (5.4%)
Experienced abnormal vaginal discharge in the past			0.057	
No	4 (28.6%)	14 (60.9%)		18 (48.6%)
Yes	10 (71.4%)	9 (39.1%)		19 (51.4%)
Was previously treated for sexually transmitted infections			0.835	
No	9 (64.3%)	14 (60.9%)		23 (62.2%)
Yes	5 (35.7%)	9 (39.1%)		14 (37.8%)

4.7. Symptoms associated with BV across genotypes and BV states

The median age (Q1-Q3) of the women in the BV intermediate group was 26.5 (21.3-28.8) and the median age (Q1-Q3) of the women in the BV positive group was 26.0 (24.0-30.5). Amongst the BV intermediate and positive groups, a higher percentage of the women did not present with symptoms of abnormal vaginal discharge (i.e. were asymptomatic). For BV intermediate and positive women harbouring GT1, there was no significant difference in women who reported abnormal vaginal discharge when compared to women who did not report the discharge ($p>0.05$) (Figure 14a).



Similarly, for the women harbouring GT2, there was no significant difference in the BV positive women who reported abnormal vaginal discharge when compared to women who did not report discharge ($p>0.05$). All BV intermediate women with GT2 reported no symptoms of abnormal vaginal discharge (Figure 14b).

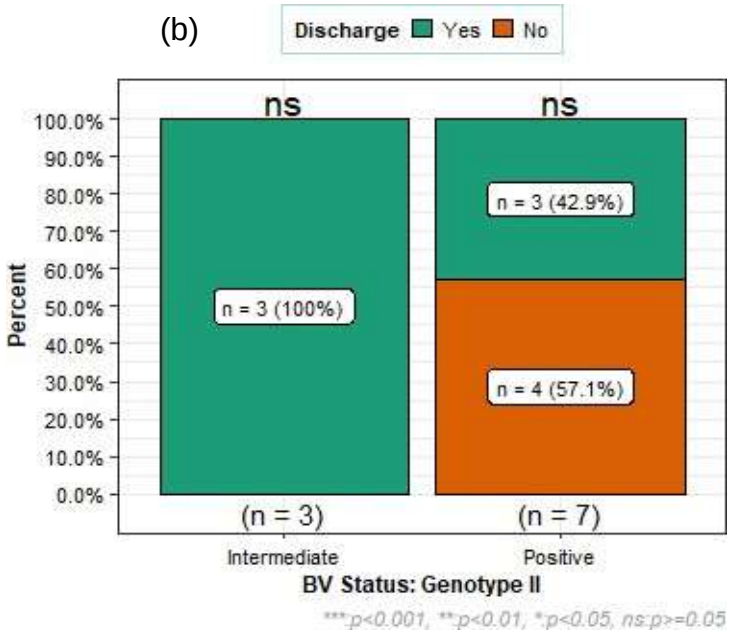


Figure 14: Symptoms of bacterial vaginosis across the intermediate and positive women in relation to genotypes. ns= refers to not significant.

CHAPTER 5

DISCUSSION

Gardnerella vaginalis is one of the most frequently isolated microorganisms from women presenting with symptoms of bacterial vaginosis [119]. High microbial loads of *G. vaginalis* in the vaginal tract has been linked to reproductive health issues such as infertility and preterm labour [9, 120]. The pathogenesis of *G. vaginalis* in the vaginal tract is not completely understood since this microorganism has been shown to be present across the BV groups (BV negative, intermediate and positive). Differentiation of *G. vaginalis* strains and subgroups according to sequence variations in the 16S rRNA gene has been made possible using molecular biology approaches, as reported by Balashov *et al.*, 2014 [21].

In this study, the diversity of the *G. vaginalis* 16S rRNA gene was analysed across BV intermediate and positive pregnant women who were diagnosed by the Nugent method. A BV negative group was not included in the diversity analysis since none of the BV negative samples produced a positive PCR amplicon for the 16S rRNA *gene* specific for *G. vaginalis*. However, the presence of *Lactobacillus crispatus* was shown to be present in the negative specimens eliminating the possibility of a failed DNA extraction or PCR amplification for the negative specimens (data not shown in this study). Our failure to amplify the *G. vaginalis* 16S rRNA gene in the negative samples differ from previously published works which had shown the presence of *G. vaginalis* in BV negative specimens based on PCR detection of the 16S rRNA gene [21, 113]. Our assumption is validated by an earlier study conducted by Verhelst *et al.*, 2004 [121] which used ARDRA in order to assess the diversity of the vaginal microbiome. In that study, ARDRA failed to identify *G. vaginalis* in women who were classified as BV

negative, however *G. vaginalis* was detected in women who were BV positive. Despite the suggested limitation, ARDRA has been useful in identifying different *G. vaginalis* genotypes [22, 97, 122].

A possible reason for the conflicting evidence between the present study and the findings of Balashov *et al.,* 2014 [21] and Janulaitiene *et al.,* 2017 [113] could be due to the following: the population studied for the Balashov [21] and Janulaitiene [113] studies included non-pregnant women from the United States and Lithuania. Geographical location, ethnic diversity and pregnancy could be a contributing factor to shifts in the vaginal microbiome leading to the observed conflicting results. However, we were unable to confirm this statement through the current study since we only tested pregnant women from one region in South Africa. This now opens up the scope for future research work on diversity assessments for *G. vaginalis* in a broader South African population.

The characteristics of the population from which successful genotyping data was obtained was as follows: the vast majority of the study population were unmarried [123] and attended high school, reaffirming the findings of a study conducted by Abbai *et al*., 2013 [124] which reported that young women with lower education are at high risk of multiple STIs. In addition, this study also revealed that a higher proportion of women had a regular sex partner, did not co-habit with their sex partner [123], had sex for the first time between the ages of 15 and 20 [124, 125], had 2-4 lifetime sex partners, said their partner has other partners [50], used condoms only "sometimes", did not use a condom during their last sexual act [126], did not smoke or consume alcohol [48], did not use intravaginal practices [127], and were in the third trimester of pregnancy. Past preterm delivery, past miscarriage, past spontaneous abortion, or past treatment for STIs, was not reported by most of the study participants. Within the overall population, clinical symptoms such as current abnormal vaginal discharge was not reported by majority of the women, whilst past abnormal vaginal discharge was.

In this study, marital status, having a regular sex partner, condom use and past abnormal vaginal discharge was significantly associated with BV status. According to a study by Abbai *et al.*, 2015 [10], clinically, it has been reported that BV represents the main cause of abnormal vaginal discharge in women of reproductive age [128]. A systematic review and meta-analysis which investigated the association between sexual risk factors and BV have suggested that BV may be sexually transmitted since many studies have shown that BV positive individuals have been associated with having a high number of lifetime or recent sexual partners, having an early age of sexual debut [124, 125] and lack of condom use during sex acts [126].

Despite being the first micro-organism associated with BV by Gardner and Dukes, *G. vaginalis* remains the most controversial species, with disparate phenotypic and genetic features, as well as an elusive role in BV pathogenesis [45], as validated by its presence across the BV groups (BV negative, intermediate and positive).

Genomic sequencing has shown differences in virulence factors among strains of *G. vaginalis* [83]. Recent comparative genomic studies indicate that *G. vaginalis* comprises at least four distinct phylogenetic clades/subtypes [102, 103]. More recently, the advent of culture-independent methods for determining the composition of the vaginal microbiome, based on whole-genome sequencing, has provided an unprecedented opportunity to investigate the diversity of *Gardnerella* spp. [129], following the report by Ahmed *et al.*, 2012 [102] who found the degree of diversity among the strains to be exceptionally high for a single species.
As mentioned previously, differentiation of *G. vaginalis* strains and subgroups according to sequence variations in the 16S rRNA gene has been made possible using molecular biology approaches [21].

Based on the ARDRA technique used in this study, restriction digestion with *Taq*I revealed the presence of two different genotypes i.e. GT1 and GT2. A third genotype (suggested to be the composite of genotypes 1 and 2) [22, 97] was not identified in this study. The absence of genotype 3 may be explained by the limited number of *G. vaginalis* strains examined in this study [23]. Similarly, a study by Pleckaityte *et al.*, 2012 [23] reported on the presence of GT1 and GT2 in a population of women with BV in Lithuania. However, the Lithuanian study was unable to detect specific subtypes associated with GT1. All the GT1 sequences in their population of women were identical. However, the present study, observed different subtypes associated with GT1. This suggests that a level of diversity does exist between *G. vaginalis* present in different geographical locations as well as across different population groups (pregnant versus non-pregnant). Additionally, the present study observed a difference in the prevalence of the different subtypes across BV intermediate and positive women. Among the BV positive women, the most prevalent subtype was 2B whereas in the BV intermediate women, the most prevalent subtype was subtype 1, thereby hypothesizing a level of genetic differences across BV intermediate and positive women. Our hypothesis was confirmed by the phylogenetic analysis which showed the presence of 5 sequence clusters on the tree indicating genetic differences across the sequences.

The study further investigated the link between genotypes and clinical symptoms of abnormal vaginal discharge. Amongst the BV intermediate and positive groups, a higher percentage of the women did not present with symptoms of abnormal vaginal discharge (i.e. were asymptomatic). This study found no significant association between genotypes harboured and symptoms of abnormal vaginal discharge. Similarly, a study by Ingianni *et al.*, 1997 [22] reported no association between genotypes of *G. vaginalis* and clinical symptoms of BV. This finding was also published by other researchers confirming that *G. vaginalis* strains involved in BV did not seem to belong to any particular subtype [107, 108]. However, a study conducted

by Santiago *et al.*, 2011 [97] showed GT2 to be the most prevalent genotype associated with symptomatic BV.

The limitations of the study are as follows: the sample size used for the analysis was small. However, despite the small sample size the study was able to provide data on the prevalent genotypes and subtypes of *G. vaginalis* in South African pregnant women across BV intermediate and positive groups, an area of research which has not been previously investigated in our setting. The study lacked a control group of non-pregnant women which would have been useful to draw comparisons regarding the distribution of the genotypes. This study did not attempt to culture *G. vaginalis* from vaginal swabs in order to perform the genotyping assays on pure cultures that would have enabled direct comparisons with previously published studies. Nevertheless, diversity assessments performed from the non-cultured clinical specimens in this study, had still provided substantial evidence. Lastly, due to the cross-sectional nature of the study, we did not associate the genotypes with pregnancy outcomes and acquisition of other infections such as HIV and genital infections. All the limitations described here will be addressed in a study that is planned for commencement in 2021.

CHAPTER 6

CONCLUSION

Given the role of *G. vaginalis* in the polymicrobial syndrome of BV, progression of the clinical symptoms of the disease, adverse pregnancy outcomes, and more significantly, its controversial presence across the BV states, it remains vital to understand its pathogenesis at genetic levels [4, 12]. This study provides the first report on the most prevalent genotypes and subtypes of *G. vaginalis* across BV intermediate and positive South African pregnant women. Restriction analysis revealed the presence of two different genotypes i.e. GT1 and GT2 as well as four subtypes (1, 2B, 2AB and 2C) circulating in our population. In addition, no significant association between reported symptoms of discharge and genotype harboured, was found. The observed diversity can be used as a foundation for future studies which aim to understand the pathogenesis of *G. vaginalis* across BV groups in women from different populations.

REFERENCES

1. Vitali, B., Abruzzo, A. and Mastromarino, P., 2017. Management of Disease and Disorders by Prebiotics and Probiotic Therapy: Probiotics in Bacterial Vaginosis. In *The Microbiota in Gastrointestinal Pathophysiology* (pp. 399-407). Academic Press.
2. Donders, G.G., 2007. Definition and classification of abnormal vaginal flora. *Best Practice & Research Clinical Obstetrics & Gynaecology*, 21(3), pp. 355-373.
3. Amsel, R., Totten, P.A., Spiegel, C.A., Chen, K.C., Eschenbach, D. and Holmes, K.K., 1983. Nonspecific vaginitis: diagnostic criteria and microbial and epidemiologic associations. *The American journal of medicine*, 74(1), pp.14-22.
4. Onderdonk, A.B., Delaney, M.L. and Fichorova, R.N., 2016. The human microbiome during bacterial vaginosis. *Clinical microbiology reviews*, 29(2), pp.223-238.
5. Kaida, A., Dietrich, J.J., Laher, F., Beksinska, M., Jaggernath, M., Bardsley, M., Smith, P., Cotton, L., Chitneni, P., Closson, K. and Lewis, D.A., 2018. A high burden of asymptomatic genital tract infections undermines the syndromic management approach among adolescents and young adults in South Africa: implications for HIV prevention efforts. *BMC infectious diseases*, 18(1), p.499.
6. Muzny, C.A and Schwebke, J.R., 2013. *Gardnerella vaginalis*: still a prime suspect in the pathogenesis of bacterial vaginosis. *Current Infectious Disease Reports*, 15(2), pp.130-135.

7. Josey, W.E. and Schwebke, J.R., 2008. The polymicrobial hypothesis of bacterial vaginosis causation: a reassessment. *International Journal of STD & AIDS*, 19(3), pp. 152-154.

8. Marrazzo, J.M., Thomas, K.K., Fiedler, T.L., Ringwood, K. and Fredricks, D.N., 2010. Risks for acquisition of bacterial vaginosis among women who report sex with women: a cohort study. *PloS one*, 5(6).

9. Menard, J.P., Mazouni, C., Salem-Cherif, I., Fenollar, F., Raoult, D., Boubli, L., Gamerre, M. and Bretelle, F., 2010. High vaginal concentrations of *Atopobium vaginae* and *Gardnerella vaginalis* in women undergoing preterm labor. *Obstetrics & Gynecology*, 115(1), pp.134–140.

10. Abbai, N.S., Reddy, T. and Ramjee, G., 2015. Prevalent bacterial vaginosis infection–a risk factor for incident sexually transmitted infections in women in Durban, South Africa. *International journal of STD & AIDS*, 27(14), pp.1283-1288.

11. Atashili, J., Poole, C., Ndumbe, P.M., Adimora, A.A. and Smith, J.S., 2008. Bacterial vaginosis and HIV acquisition: a meta-analysis of published studies. *AIDS (London, England)*, 22(12), p.1493.

12. Turovskiy, Y., Sutyak Noll, K. and Chikindas, M.L., 2011. The aetiology of bacterial vaginosis. *Journal of applied microbiology*, 110(5), pp.1105-1128.

13. Morrill, S., Gilbert, N.M. and Lewis, A.L., 2020. *Gardnerella vaginalis* as a Cause of Bacterial Vaginosis: Appraisal of the Evidence From in vivo Models. *Frontiers in Cellular and Infection Microbiology*, 10, p168.

14. Schellenberg, J.J, Patterson, M.H. and Hill, J.E., 2017. *Gardnerella vaginalis* diversity and ecology in relation to vaginal symptoms. *Research in microbiology*, 168(9-10), pp.837-844.

15. Srinivasan, S., Hoffman, N.G., Morgan, M.T., Matsen, F.A., Fiedler, T.L., Hall,

R.W., Ross, F.J., McCoy, C.O., Bumgarner, R., Marrazzo, J.M. and Fredricks, D.N., 2012. Bacterial communities in women with bacterial vaginosis: high resolution phylogenetic analyses reveal relationships of microbiota to clinical criteria. *PloS one*, 7(6).

16. Patterson, J.L., Stull-Lane, A., Girerd, P.H. and Jefferson, K.K., 2010. Analysis of adherence, biofilm formation and cytotoxicity suggests a greater virulence potential of *Gardnerella vaginalis* relative to other bacterial-vaginosis-associated anaerobes. *Microbiology*, 156(Pt 2), p.392.

17. Castro. J., Jefferson, K.K. and Cerca, N., 2020. Genetic heterogeneity and taxonomic diversity among *Gardnerella* species. *Trends in microbiology*, 28(3), pp.202-211.

18. Janulaitiene, M., Gegzna, V., Baranauskiene, L., Bulavaitė, A., Simanavicius, M. and Pleckaityte, M., 2018. Phenotypic characterization of *Gardnerella vaginalis* subgroups suggests differences in their virulence potential. *PloS one*, 13(7).

19. Shipitsyna, E., Krysanova, A., Khayrullina, G., Shalepo, K., Savicheva, A., Guschin, A. and Unemo, M., 2019. Quantitation of all four *Gardnerella vaginalis* clades detects abnormal vaginal microbiota characteristic of bacterial vaginosis more accurately than putative *G. vaginalis* sialidase A gene count. *Molecular diagnosis & therapy*, 23(1), pp.139-147.

20. Vaneechoutte, M., De Beenhouwer, H., Claeys, G., Verschraegen, G.E.R.D.A., De Rouck, A., Paepe, N., Elaichouni, A.B.D.E.S.L.A.M. and Portaels, F., 1993. Identification of Mycobacterium species by using amplified ribosomal DNA restriction analysis. *Journal of Clinical Microbiology*, 31(8), pp.2061-2065.

21. Balashov, S.V., Mordechai, E., Adelson, M.E. and Gygax, S.E., 2014.

Identification, quantification and subtyping of *Gardnerella vaginalis* in noncultured clinical vaginal samples by quantitative PCR. *Journal of medical microbiology*, 63(2), pp.162-175.

22. Ingianni, A., Petruzzelli, S., Morandotti, G. and Pompei, R., 1997. Genotypic differentiation of *Gardnerella vaginalis* by amplified ribosomal DNA restriction analysis (ARDRA). *FEMS Immunology & Medical Microbiology*, 18(1), pp.61-66.
23. Pleckaityte, M., Janulaitiene, M., Lasickiene, R. and Zvirbliene, A., 2012. Genetic and biochemical diversity of *Gardnerella vaginalis* strains isolated from women with bacterial vaginosis. *FEMS Immunology & Medical Microbiology*, 65(1), pp. 69–77.
24. Dessai, F., Nyirenda, M., Sebitloane, M. and Abbai, N., 2020. Diagnostic evaluation of the BD Affirm VPIII assay as a point-of-care test for the diagnosis of bacterial vaginosis, trichomoniasis and candidiasis. *International journal of STD & AIDS*, 31(4), pp.303-311.
25. Sobel, J.D., 2017. Vaginitis, vulvitis, cervicitis and cutaneous vulval lesions. *In Infectious diseases* (pp. 483-491). Elsevier.
26. Dingens, A.S., Fairfortune, T.S., Reed, S. and Mitchell, C., 2016. Bacterial vaginosis and adverse outcomes among full-term infants: a cohort study. *BMC Pregnancy Childbirth*, 16(1), p.278.
27. Karlowsky, J.A., Lagacé-Wiens, P.R., Simner, P.J., DeCorby, M.R., Adam, H.J., Walkty, A., Hoban, D.J. and Zhanel, G.G., 2011. Antimicrobial resistance in urinary tract pathogens in Canada from 2007 to 2009: CANWARD surveillance study. *Antimicrobial agents and chemotherapy*, 55(7), pp.3169-3175.

28. Lewis, W.G., Robinson, L.S., Perry, J.C., Lewis, A.L., 2013. Degradation, foraging, and depletion of mucus sialoglycans by the vagina-adapted Actinobacterium *Gardnerella vaginalis*. *Journal of Biological Chemistry*, 288(17), pp. 12067–79.

29. Myer, L., Denny, L., Telerant, R., de Souza, M., Wright Jr, T.C. and Kuhn, L., 2005. Bacterial vaginosis and susceptibility to HIV infection in South African women: a nested case-control study. *The Journal of infectious diseases*, 192(8), pp.1372-1380.

30. Kenyon, C., Colebunders, R. and Crucitti, T., 2013. The global epidemiology of bacterial vaginosis: a systematic review. *American journal of obstetrics and gynecology*, 209(6), pp.505-523.

31. Bayigga, L., Kateete, D.P., Anderson, D.J., Sekikubo, M. and Nakanjako, D., 2019. Diversity of vaginal microbiota in sub-Saharan Africa and its effects on HIV transmission and prevention. *American journal of obstetrics and gynecology*, 220(2), pp.155-166.

32. Jespers, V., Crucitti, T., Menten, J., Verhelst, R., Mwaura, M., Mandaliya, K., Ndayisaba, G.F., Delany-Moretlwe, S., Verstraelen, H., Hardy, L. and Buvé, A., 2014. Prevalence and correlates of bacterial vaginosis in different sub-populations of women in sub-Saharan Africa: a cross-sectional study. *PLoS one*, 9(10).

33. Torrone, E.A., Morrison, C.S., Chen, P.L., Kwok, C., Francis, S.C., Hayes, R.J., Looker, K.J., McCormack, S., McGrath, N., van de Wijgert, J.H. and Watson-Jones, D., 2018. Prevalence of sexually transmitted infections and bacterial vaginosis among women in sub-Saharan Africa: An individual participant data meta-analysis of 18 HIV prevention studies. *PLoS medicine*, 15(2), p.e1002511.

34. Johnson, L.F., Dorrington, R.E., Bradshaw, D. and Coetzee, D.J., 2011. The effect of syndromic management interventions on the prevalence of sexually transmitted infections in South Africa. *Sexual & Reproductive Healthcare*, 2(1), pp.13-20.

35. Nel, A., Mabude, Z., Smit, J., Kotze, P., Arbuckle, D., Wu, J., van Niekerk, N. and van de Wijgert, J., 2012. HIV incidence remains high in KwaZulu-Natal, South Africa: evidence from three districts. *PLoS One*, 7(4).

36. Ramjee, G., Williams, B., Gouws, E., van Dyck, E., De Deken, B. and Karim, S.A., 2005. The impact of incident and prevalent *herpes simplex virus*-2 infection on the incidence of HIV-1 infection among commercial sex workers in South Africa. *JAIDS Journal of Acquired Immune Deficiency Syndromes*, 39(3), pp.333-339.

37. Frolich, J.A., Abdool Karim, Q., Mashego, M.M., Sturm, A.W. and Abdool Karim, S.S., 2007. Opportunities for treating sexually transmitted infections and reducing HIV risk in rural South Africa. *Journal of advanced nursing*, 60(4), pp.377-383.

38. Hoque, M.E., 2011. Reported risky sexual practices amongst female undergraduate students in KwaZulu-Natal, South Africa. *African Journal of Primary Health Care & Family Medicine*, 3(1).

39. Yen, S., Shafer, M.A., Moncada, J., Campbell, C.J., Flinn, S.D. and Boyer, C.B., 2003. Bacterial vaginosis in sexually experienced and non–sexually experienced young women entering the military. *Obstetrics & Gynecology*, 102(5), pp.927-933.

40. Hutchinson, K.B., Kip, K.E. and Ness, R.B., 2007. Vaginal douching and development of bacterial vaginosis among women with normal and abnormal vaginal microflora. *Sexually transmitted diseases*, 34(9), pp.671-675.

41. Cherpes, T.L., Hillier, S.L., Meyn, L.A., Busch, J.L. and Krohn, M.A., 2008. A delicate balance: risk factors for acquisition of bacterial vaginosis include sexual activity, absence of hydrogen peroxide-producing lactobacilli, black race, and

positive *herpes simplex virus* type 2 serology. *Sexually transmitted diseases*, 35(1), pp.78-83.

42. Verstraelen, H., 2008. Bacterial vaginosis: a sexually enhanced disease. *International journal of STD & AIDS*, 19(8), pp.575-576.

43. Fethers, K.A., Fairley, C.K., Morton, A., Hocking, J.S., Hopkins, C., Kennedy, L.J., Fehler, G. and Bradshaw, C.S., 2009. Early sexual experiences and risk factors for bacterial vaginosis. *The Journal of infectious diseases*, 200(11), pp.1662-1670.

44. Verstraelen, H., Verhelst, R., Vaneechoutte, M. and Temmerman, M., 2010. The epidemiology of bacterial vaginosis in relation to sexual behaviour. *BMC infectious diseases*, 10(1), p.81.

45. Gardner, H.L. and Dukes, C.D., 1955. *Haemophilus vaginalis* vaginitis: a newly defined specific infection previously classified "nonspecific" vaginitis. *American journal of obstetrics and gynecology*, 69(5), pp.962-976.

46. Criswell, B.S., Ladwig, C.L., Gardner, H.L. and Dukes, C.D., 1969. *Haemophilus vaginalis*: vaginitis by inoculation from culture. *Obstetrics & Gynecology*, 33(2), pp.195-199.

47. Gardner, H.L., 1980. *Haemophilus vaginalis* vaginitis after twenty-five years. *American journal of obstetrics and gynecology*, 137(3), pp.385-391.

48. Francis, S.C., Looker, C., Vandepitte, J., Bukenya, J., Mayanja, Y., Nakubulwa, S., Hughes, P., Hayes, R.J., Weiss, H.A. and Grosskurth, H., 2016. Bacterial vaginosis among women at high risk for HIV in Uganda: high rate of recurrent diagnosis despite treatment. *Sex Transm Infect*, 92(2), pp.142-148.

49. Nagot, N., Ouedraogo, A., Defer, M.C., Vallo, R., Mayaud, P. and Van de Perre, P., 2007. Association between bacterial vaginosis and *Herpes simplex virus* type-2

infection: implications for HIV acquisition studies. *Sexually transmitted infections*, 83(5), pp.365-368.

50. Abbai, N.S., Nyirenda, M., Naidoo, S. and Ramjee, G., 2018. Prevalent *herpes simplex virus*-2 increases the risk of incident bacterial vaginosis in women from South Africa. *AIDS and behavior*, pp.1-9.
51. van de Wijgert, J.H., 2017. The vaginal microbiome and sexually transmitted infections are interlinked: consequences for treatment and prevention. *PLoS medicin*e, 14(12).
52. Haggerty, C.L., Hillier, S.L., Bass, D.C., Ness, R.B. and PID Evaluation and Clinical Health (PEACH) Study Investigators, 2004. Bacterial vaginosis and anaerobic bacteria are associated with endometritis. *Clinical Infectious Diseases*, 39(7), pp.990-995.
53. Lamont, R.F., Sobel, J.D., Akins, R.A., Hassan, S.S., Chaiworapongsa, T., Kusanovic, J.P. and Romero, R., 2011. The vaginal microbiome: new information about genital tract flora using molecular based techniques. BJOG: *An International Journal of Obstetrics & Gynaecology*, 118(5), pp.533-549.
54. Machado, D., Castro, J., Martinez-de-Oliveira, J., Nogueira-Silva, C. and Cerca, N., 2017. Prevalence of bacterial vaginosis in Portuguese pregnant women and vaginal colonization by *Gardnerella vaginalis*. *PeerJ*, 5, p.e3750.
55. Grether, J.K. and Nelson, K.B., 2000. Possible decrease in prevalence of cerebral palsy in premature infants. *The journal of Pediatrics*, 136(1), p.133.
56. Mann, J.R., Mcdermott, S., Bao, H. and Bersabe, A., 2009. Maternal genitourinary infection and risk of cerebral palsy. *Developmental Medicine and Child Neurology*, 51(4), pp.282-288.

57. Boyle, A.K., Rinaldi, S.F., Norman, J.E. and Stock, S.J., 2017. Preterm birth: Inflammation, fetal injury and treatment strategies. *Journal of reproductive immunology*, 119, pp.62-66.

58. Mastromarino, P., Hemalatha, R., Barbonetti, A., Cinque, B., Cifone, M.G., Tammaro, F. and Francavilla, F., 2014. Biological control of vaginosis to improve reproductive health. *The Indian journal of medical research*, 140(Suppl 1), p.S91.

59. Schwebke, J.R., Hillier, S.L., Sobel, J.D., McGregor, J.A. and Sweet, R.L., 1996. Validity of the vaginal gram stain for the diagnosis of bacterial vaginosis. *Obstetrics & gynecology*, 88(4), pp.573-576.

60. Beverly, E.S., Chen, H.Y., Wang, Q.J., Zariffard, M.R., Cohen, M.H. and Spear, G.T., 2005. Utility of Amsel criteria, Nugent score, and quantitative PCR for *Gardnerella vaginalis*, *Mycoplasma hominis*, and *Lactobacillus* spp. for diagnosis of bacterial vaginosis in human immunodeficiency virus-infected women. *Journal of clinical microbiology*, 43(9), pp.4607-4612.

61. Menard, J.P., Fenollar, F., Henry, M., Bretelle, F. and Raoult, D., 2008. Molecular quantification of *Gardnerella vaginalis* and *Atopobium vaginae* loads to predict bacterial vaginosis. *Clinical Infectious Diseases*, 47(1), pp.33-43.

62. Coleman, J.S. and Gaydos, C.A., 2018. Molecular diagnosis of bacterial vaginosis: an update. *Journal of clinical microbiology*, 56(9), pp.e00342-18.

63. Workowski, K.A. and Berman, S.M., 2006. *Sexually transmitted diseases treatment guidelines*, 2006.

64. Nugent, R.P., Krohn, M.A. and Hillier, S.L., 1991. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. *Journal of clinical microbiology*, 29(2), pp.297-301.

65. Amegashie, C.P., Gilbert, N.M., Peipert, J.F., Allsworth, J.E., Lewis, W.G. and Lewis, A.L., 2017. Relationship between nugent score and vaginal epithelial exfoliation. *PLoS one*, 12(5).

66. Leopold, S., 1953. Heretofore undescribed organism isolated from the genitourinary system. *United States Armed Forces Medical Journal*, 4(2), pp.263-6.

67. Catlin, B.W., 1992. *Gardnerella vaginalis*: characteristics, clinical considerations, and controversies. *Clinical Microbiology Reviews*, 5(3), pp.213-237.

68. Zinnemann, K. and Turner, G.C., 1963. The taxonomic position of "*Haemophilus vaginalis*"[*Corynebacterium vaginale*]. *The Journal of Pathology and Bacteriology*, 85(1), pp.213-219.

69. Deane, C., Smith, C.D., Fykes, T. and Sampson, C.C., 1972. *Corynebacterium vaginale*. An analysis of 68 isolations. *The Medical annals of the District of Columbia*, 41(1), p.4.

70. Greenwood, J.R. and Pickett, M.J., 1980. Transfer of *Haemophilus vaginalis* Gardner and Dukes to a New Genus, *Gardnerella*: *G. vaginalis* (Gardner and Dukes) comb. nov. *International Journal of Systematic and Evolutionary Microbiol*ogy, 30(1), pp.170-178.

71. Piot, P., Van Dyck, E.D.D.Y., Goodfellow, M. and Falkow, S., 1980. A taxonomic study of *Gardnerella vaginalis* (*Haemophilus vaginalis*) Gardner and Dukes 1955. *Microbiology*, 119(2), pp.373-396.

72. Chart, H., 2012. *Vibrio, mobiluncus, gardnerella* and *spirillum*: Cholera; vaginosis; rat bite fever. In *Medical Microbiology* (pp.314-323). Churchill Livingstone.

73. Wong, Y.P., Tan, G.C., Wong, K.K., Anushia, S. and Cheah, F., 2018. *Gardnerella vaginalis* in perinatology: An overview of the clinicopathological correlation. *The Malaysian journal of pathology*, 40(3), pp.267-286.

74. Lasa, I. and Penadés, J.R., 2006. Bap: a family of surface proteins involved in biofilm formation. *Research in microbiology*, 157(2), pp.99-107.

75. Davies, P.J., Cornwell, M.M., Johnson, J.D., Reggianni, A., Myers, M. and Murtaugh, M.P., 1984. Studies on the effects of dansylcadaverine and related compounds on receptor-mediated endocytosis in cultured cells. *Diabetes Care*, 7(Suppl 1), pp.35-41.

76. Cereija, T.B., Castro, J., Alves, P. and Cerca, N., 2013. Influence of anaerobic conditions on vaginal microbiota recovery from bacterial vaginosis patients. *Sex Transm Infect*, 89(4), pp.307-307.

77. Bratcher, D.F., 2018. Other Gram-Positive Bacilli. *In Principles and Practice of Pediatric Infectious Diseases* (pp. 786-790). Elsevier.

78. Harwich, M.D., Alves, J.M., Buck, G.A., Strauss, J.F., Patterson, J.L., Oki, A.T., Girerd, P.H. and Jefferson, K.K., 2010. Drawing the line between commensal and pathogenic *Gardnerella vaginalis* through genome analysis and virulence studies. *BMC genomics*, 11(1), p.375.

79. Griffiths, E., 1991. Iron and bacterial virulence—a brief overview. *Biology of metals*, 4(1), pp.7-13.

80. Jarosik, G.P., Land, C.B., Duhon, P., Chandler, R. and Mercer, T., 1998. Acquisition of iron by Gardnerella vaginalis. *Infection and immunity*, 66(10), pp.5041-5047.

81. Rosa, L., Cutone, A., Lepanto, M.S., Paesano, R. and Valenti, P., 2017. Lactoferrin:

a natural glycoprotein involved in iron and inflammatory homeostasis. *International journal of molecular sciences*, 18(9), p.1985.

82. LaRocca, T.J., Stivison, E.A., Hod, E.A., Spitalnik, S.L., Cowan, P.J., Randis, T.M. and Ratner, A.J., 2014. Human-specific bacterial pore-forming toxins induce programmed necrosis in erythrocytes. *MBio*, 5(5), pp.e01251-14.

83. Yeoman, C.J., Yildirim, S. and Thomas, S.M., 2010. Durkin a. S, Torralba M, Sutton G, et al. Comparative genomics of *Gardnerella vaginalis* strains reveals substantial differences in metabolic and virulence potential. *PLoS One*, 5(8), p.e12411.

84. Mitchell, C., Fredricks, D., Agnew, K. and Hitti, J., 2015. Hydrogen-peroxide producing lactobacilli are associated with lower levels of vaginal IL 1β, independent of bacterial vaginosis. *Sexually transmitted diseases*, 42(7), p.358.

85. Shipitsyna, E., Roos, A., Datcu, R., Hallén, A., Fredlund, H., Jensen, J.S., Engstrand, L. and Unemo, M., 2013. Composition of the vaginal microbiota in women of reproductive age–sensitive and specific molecular diagnosis of bacterial vaginosis is possible?. *PLoS one*, 8(4).

86. Hickey, R.J. and Forney, L.J., 2014. *Gardnerella vaginalis* does not always cause bacterial vaginosis. *The Journal of infectious diseases*, 210(10), pp.1682-1683.

87. Castro, J., Alves, P., Sousa, C., Cereija, T., França, Â., Jefferson, K.K. and Cerca, N., 2015. Using an in-vitro biofilm model to assess the virulence potential of bacterial vaginosis or non-bacterial vaginosis *Gardnerella vaginalis* isolates. *Scientific reports*, 5, p.11640.

88. Machado, A., Jefferson, K.K. and Cerca, N., 2013. Interactions between *Lactobacillus crispatus* and bacterial vaginosis (BV)-associated bacterial species in

initial attachment and biofilm formation. *International journal of molecular sciences*, 14(6), pp.12004-12012.

89. Alves, P., Castro, J., Sousa, C., Cereija, T.B. and Cerca, N., 2014. *Gardnerella vaginalis* outcompetes 29 other bacterial species isolated from patients with bacterial vaginosis, using in an in vitro biofilm formation model. *The Journal of infectious diseases*, 210(4), pp.593-596.

90. Hardy, L., Cerca, N., Jespers, V., Vaneechoutte, M. and Crucitti, T., 2017. Bacterial biofilms in the vagina. *Research in microbiology*, 168(9-10), pp.865-874.

91. Swidsinski, A., Mendling, W., Loening-Baucke, V., Ladhoff, A., Swidsinski, S., Hale, L.P. and Lochs, H., 2005. Adherent biofilms in bacterial vaginosis. *Obstetrics & Gynecology*, 106(5), pp.1013-1023.

92. Tomusiak, A., Heczko, M. and Bogumił, P., 2011. Antibiotic resistance of *Gardnerella vaginalis* isolated from cases of bacterial vaginosis. *Ginekologia polska*, 82(12).

93. Machado, D., Castro, J., Palmeira-de-Oliveira, A., Martinez-de-Oliveira, J. and Cerca, N., 2016. Bacterial vaginosis biofilms: challenges to current therapies and emerging solutions. *Frontiers in microbiology*, 6, p.1528.

94. Gelber, S.E., Aguilar, J.L., Lewis, K.L. and Ratner, A.J., 2008. Functional and phylogenetic characterization of Vaginolysin, the human-specific cytolysin from *Gardnerella vaginalis*. *Journal of bacteriology*, 190(11), pp.3896-3903.

95. Zilnyte, M., Venclovas, Č., Zvirbliene, A., and Pleckaityte, M., 2015. The cytolytic activity of vaginolysin strictly depends on cholesterol and is potentiated by human CD59. *Toxins*, 7(1), pp.110–128.

96. Briselden, A.M., Moncla, B.J., Stevens, C.E. and Hillier, S.L., 1992. Sialidases (neuraminidases) in bacterial vaginosis and bacterial vaginosis-associated microflora. *Journal of clinical microbiology*, 30(3), pp.663-666.

97. dos Santos Santiago, G.L., Deschaght, P., El Aila, N., Kiama, T.N., Verstraelen, H., Jefferson, K.K., Temmerman, M. and Vaneechoutte, M., 2011. *Gardnerella vaginalis* comprises three distinct genotypes of which only two produce sialidase. *American journal of obstetrics and gynecology*, 204(5), pp.450-e1.

98. Lewis, A.L. and Lewis, W.G., 2012. Host sialoglycans and bacterial sialidases: a mucosal perspective. *Cellular microbiology*, 14(8), pp.1174-1182.

99. Cauci, S., Culhane, J.F., Di Santolo, M. and McCollum, K., 2008. Among pregnant women with bacterial vaginosis, the hydrolytic enzymes sialidase and prolidase are positively associated with interleukin-1β. *American journal of obstetrics and gynecology*, 198(1), pp.132.e1.

100. Schwebke, J.R., Muzny, C.A., and Josey, W.E., 2014. Role of *Gardnerella vaginalis* in the pathogenesis of bacterial vaginosis: a conceptual model. *The Journal of infectious diseases*, 210(3), pp.338-343.

101. Lim, D., Trivedi, H. and Nath, K., 1994. Determination of *Gardnerella vaginalis* genome size by pulsed-field gel electrophoresis. *DNA Research*, 1(3), pp.115-122.

102. Ahmed, A., Earl, J., Retchless, A., Hillier, S.L., Rabe, L.K., Cherpes, T.L., Powell, E., Janto, B., Eutsey, R., Hiller, N.L. and Boissy, R., 2012. Comparative genomic analyses of 17 clinical isolates of *Gardnerella vaginalis* provide evidence of multiple genetically isolated clades consistent with subspeciation into genovars. *Journal of bacteriology*, 194(15), pp.3922-3937.

103. Malki, K., Shapiro, J.W., Price, T.K., Hilt, E.E., Thomas-White, K., Sircar, T.,

Rosenfeld, A.B., Kuffel, G., Zilliox, M.J., Wolfe, A.J. and Putonti, C., 2016. Genomes of *gardnerella* strains reveal an abundance of prophages within the bladder microbiome. *PLoS one*, 11(11).

104. Vaneechoutte, M., Guschin, A., Van Simaey, L., Gansemans, Y., Van Nieuwerburgh, F. and Cools, P., 2019. Emended description of *Gardnerella vaginalis* and description of *Gardnerella leopoldii* sp. nov., *Gardnerella piotii* sp. nov. and *Gardnerella swidsinskii* sp. nov., with delineation of 13 genomic species within the genus *Gardnerella*. *International journal of systematic and evolutionary microbiology*, 69(3), pp.679-687.

105. Piot, P., Van Dyck, E., Peeters, M., Hale, J., Totten, P.A. and Holmes, K.K., 1984. Biotypes of *Gardnerella vaginalis*. *Journal of clinical microbiology*, 20(4), pp.677-679.

106. Nath, K., Choi, D.J. and Devlin, D., 1991. The characterization of *Gardnerella vaginalis* DNA using non-radioactive DNA probes. *Research in microbiology*, 142(5), pp.573-583.

107. Nath, K., Devlin, D. and Beddoe, A.M., 1992. Heterogeneity in restriction patterns of *Gardnerella vaginalis* isolates from individuals with bacterial vaginosis. *Research in microbiology*, 143(2), pp.199-209.

108. Wu, S.R., Hillier, S.L. and Nath, K., 1996. Genomic DNA fingerprint analysis of biotype 1 *Gardnerella vaginalis* from patients with and without bacterial vaginosis. *Journal of clinical microbiology*, 34(1), pp.192-195.

109. Brotman, R.M., and Ravel, J., 2008. Ready or not: the molecular diagnosis of bacterial vaginosis.

110. McInerney, P., Adams, P. and Hadi, M.Z., 2014. Error rate comparison during polymerase chain reaction by DNA polymerase. *Molecular biology international*, 2014.

111. Jayaprakash, T.P., Schellenberg, J.J. and Hill, J.E., 2012. Resolution and characterization of distinct cpn60-based subgroups of *Gardnerella vaginalis* in the vaginal microbiota. *PLoS One*, 7(8).

112. Schellenberg, J.J., Jayaprakash, T.P., Gamage, N.W., Patterson, M.H., Vaneechoutte, M. and Hill, J.E., 2016. *Gardnerella vaginalis* subgroups defined by cpn60 sequencing and sialidase activity in isolates from Canada, Belgium and Kenya. *PLoS one*, 11(1).

113. Janulaitiene, M., Paliulyte, V., Grinceviciene, S., Zakareviciene, J., Vladisauskiene, A., Marcinkute, A. and Pleckaityte, M., 2017. Prevalence and distribution of *Gardnerella vaginalis* subgroups in women with and without bacterial vaginosis. *BMC infectious diseases*, 17(1), p.394.

114. Nazir, R., Rehman, S., Nisa, M. and ali Baba, U., 2019. Exploring bacterial diversity: from cell to sequence. *In Freshwater Microbiology* (pp. 263-306). Academic Press.

115. Bharagava, R.N., Purchase, D., Saxena, G. and Mulla, S.I., 2019. Applications of metagenomics in microbial bioremediation of pollutants: from genomics to environmental cleanup. *In Microbial diversity in the genomic era* (pp. 459-477). Academic Press.

116. Rastogi, G. and Sani, R.K., 2011. Molecular techniques to assess microbial community structure, function, and dynamics in the environment. *In Microbes and microbial technology* (pp. 29-57). Springer, New York, NY.

117. Aroutcheva, A.A., Simoes, J.A., Behbakht, K. and Faro, S., 2001. *Gardnerella vaginalis* isolated from patients with bacterial vaginosis and from patients with healthy vaginal ecosystems. *Clinical infectious diseases*, 33(7), pp.1022-1027.

118. Sanger F, 1956. Currents in Biochemical Research. New York-London, 424.

119. Schellenberg, J.J., Links, M.G., Hill, J.E., Dumonceaux, T.J., Kimani, J., Jaoko, W., Wachihi, C., Mungai, J.N., Peters, G.A., Tyler, S. and Graham, M., 2011. Molecular definition of vaginal microbiota in East African commercial sex workers. *Appl Environ Microbiol*., 77(12), pp.4066-4074.

120. Shimaoka, M., Yo, Y., Doh, K., Kotani, Y., Suzuki, A., Tsuji, I., Mandai, M. and Matsumura, N., 2019. Association between preterm delivery and bacterial vaginosis with or without treatment. *Scientific reports*, 9(1), pp.1-8.

121. Verhelst, R., Verstraelen, H., Claeys, G., Verschraegen, G., Delanghe, J., Van Simaey, L., De Ganck, C., Temmerman, M. and Vaneechoutte, M., 2004. Cloning of 16S rRNA genes amplified from normal and disturbed vaginal microflora suggests a strong association between *Atopobium vaginae*, *Gardnerella vaginalis* and bacterial vaginosis. *BMC microbiology*, 4(1), p.16.

122. Vaneechoutte, M., Rossau, R., De Vos, P., Gillis, M., Janssens, D., Paepe, N., De Rouck, A., Fiers, T., Claeys, G. and Kersters, K., 1992. Rapid identification of bacteria of the *Comamonadaceae* with amplified ribosomal DNA-restriction analysis (ARDRA). *FEMS Microbiology Letters*, 93(3), pp.227-233.

123. Naidoo, S., Wand, H., Abbai, N.S. and Ramjee, G., 2014. High prevalence and incidence of sexually transmitted infections among women living in KwaZulu-Natal, South Africa. *AIDS Research and Therapy*, 11(1), p31.

124. Abbai, N, S., Wand, H. and Ramjee, G., 2013. Sexually transmitted infections in women participating in a biomedical intervention trial in Durban: Prevalence, coinfections, and risk factors. *Journal of sexually transmitted diseases*, 2013.

125. Kenyon, C. and Badri, M., 2009. The role of concurrent sexual relationships in the spread of sexually transmitted infections in young South Africans. *Southern African Journal of HIV Medicine*, 10(1).

126. Fethers, K.A., Fairley, C.K., Hocking, J.S., Gurrin, L.C. and Bradshaw, C.S., 2008. Sexual risk factors and bacterial vaginosis: a systematic review and meta-analysis. *Clinical Infectious Diseases*, 47(11), pp.1426–1435.

127. Low, N., Chersich, M.F., Schmidlin, K., Egger, M., Francis, S.C., Van de Wijgert, J.H., Hayes, R.J., Baeten, J.M., Brown, J., Delaney-Moretlwe, S. and Kaul, R., 2011. Intravaginal practices, bacterial vaginosis, and HIV infection in women: individual participant data meta-analysis. *PLoS medicine*, 8(2).

128. Bradshaw, C.S., Morton, A.N., Hocking, J., Garland, S.M., Morris, M.B., Moss, L.M., Horvath, L.B., Kuzevska, I. and Fairley, C.K., 2006. High recurrence rates of bacterial vaginosis over the course of 12 months after oral metronidazole therapy and factors associated with recurrence. *The Journal of infectious diseases*, 193(11), pp.1478–1486.

129. Huang, B., Fettweis, J.M., Brooks, J.P., Jefferson, K.K. and Buck, G.A., 2014. The changing landscape of the vaginal microbome. *Clinics in laboratory medicine*, 34(4), pp.747-761.

APPENDIX

Appendix 1: BREC Approval letter

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

07 October 2019

Miss Kayla Pillay (219090946)
School of Clinical Medicine
Medical School Campus

Dear Miss Kayla Pillay,

Protocol reference number: BREC/00000093/2019
Project title: Genotyping of Gardnerella vaginalis from pregnant women in Durban by Amplified Ribosomal DNA Restriction Analysis
Degree Purposes: 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 07 October 2019. **Please ensure that 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 **07 October 2019.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC 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 (2006) (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 **12 November 2019.**

Yours sincerely

[redacted]

Prof V Rambiritch (Chair)

Biomedical Research Ethics Committee
Prof V Rambiritch (Chair)
UKZN Research Ethics Office Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Website: http://research.ukzn.ac.za/Research-Ethics/

Founding Campuses: Edgewood Howard College Medical School Pietermaritzburg Westville

INSPIRING GREATNESS