# Genetic diversity of *Gardnerella vaginalis* in pregnant women diagnosed with intermediate and positive bacterial vaginosis

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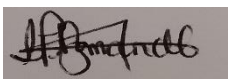
Signed: 

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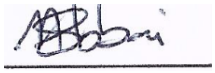

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## LIST OF ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| BREC | Biomedical Research Ethics Committee |
| UKZN | University of KwaZulu Natal |
| BV | Bacterial Vaginosis |
| HIV | Human Immuno-deficiency Virus |
| PCR | Polymerase Chain Reaction |
| DNA | Deoxyribonucleic Acid NS |
| Nugent Score rRNA | Ribosomal Ribonucleic Acid |
| STIs | Sexually Transmitted Infections |
| HSV-2 | Herpes Simplex Virus-2 |
| FDA | Food and Drug Administration |
| RNA | Ribose Nucleic Acid |
| UKZN | University of KwaZulu- Natal |
| BLAST | Basic Local Alignment Search Tool |
| NAD | Nicotinamide Adenine Dinucleotide |
| SA | South Africa |
| KZN | KwaZulu Natal |

## ABSTRACT

Bacterial vaginosis (BV) is the main cause of abnormal vaginal discharge in women of reproductive age. *Gardnerella vaginalis,* has been detected in almost all women with BV. However, there is limited information on the genetic diversity of *G. vaginalis* isolated from BV intermediate and positive cases. In this study we investigated the genetic diversity of *G. vaginalis* strains from South African pregnant women.

Vaginal swabs were characterized by the Nugent method. A total of n= 87 samples were included in the genetic analysis, (n=50 BV positive) and (n=37 BV intermediate). The presence of *G. vaginalis* was detected by PCR using bacterium specific 16S rRNA primers. All PCR positive amplicons were sequenced by the Sanger method and the edited sequence data was used for the phylogenetic analysis using the PHYLIP software. The *sialidase A* gene was amplified by PCR using specific primers and the copy numbers of *sialidase A* gene was quantified by droplet digital PCR. To assess the diversity of the *sialidase A* gene, Sanger sequencing was performed.

The 16S rRNA gene from *G. vaginalis* was amplified in all BV positive and BV intermediate samples. All PCR amplicons were successfully sequenced and the nucleotide BLAST results revealed 100% identify to *G. vaginalis.* The phylogenetic analysis revealed that there is no diversity in *G. vaginalis* present in BV positive and intermediate cases. The phylogenetic tree of *sialidase A* sequences from intermediate and positive BV cases revealed two major clades which showed differences related to *sialidase A* copy number. Quantification of *sialidase A* showed that the average number of copies per cell was much higher in the BV positive group compared to the intermediate group. Some of the intermediate cases showed high copy numbers for the virulence gene and clustered with the BV positive cases.

In the present study the 16S rRNA sequences of the *G. vaginalis* from BV intermediate and positive women showed that there is no genetic diversity in *G. vaginalis* detected in BV positive and intermediate samples. The phylogenetic tree of *sialidase A* gene sequences of intermediate and positive BV revealed two major clades which showed differences related to *sialidase A* copy number. This data was previously lacking in our setting, especially in a pregnant population. We further demonstrate for the first time that the genetic information present within the *sialidase A* gene has a direct influence on BV status.

# CHAPTER 1

# INTRODUCTION

Bacterial vaginosis (BV) is a polymicrobial syndrome that affects women of reproductive age and is the most prevalent cause of symptomatic vaginal discharge (1)**.** Bacterial vaginosis has been associated with complications of reproductive health, such as preterm birth and acquisition or transmission of sexually transmitted infections (STIs), including human immunodeficiency virus (HIV) infect ion. Facultative anaerobic microorganisms such as *Gardnerella vaginalis, Atopobium vaginae*, *Mobiluncus mulieris, Prevotella bivia*, *Peptostreptococcus* and *Bacteroides* have been associated with BV (2).

The cause of BV is not fully understood. However, it has been suggested that it is transmissible, and that *G. vaginalis* may be the main causative agent (3). Studies using enzymatic assays to define *G. vaginalis* biotypes, as well as more recent genomic comparisons of G. *vaginalis* isolates from symptomatic and asymptomatic women, suggested that some strains of *G. vaginalis* may play a key role in the pathogenesis of BV (4-6) *G. vaginalis* has appeared to be the most virulent BV associated anaerobe (7), demonstrating greater adherence to vaginal cells, cytotoxicity and biofilm producing capacity (8). These virulence factors suggest that *G. vaginalis* plays a key role in BV biofilms development.

Conventional microbial techniques are useful screening tools for the identification of women with BV. However, they do not allow the prediction of the clinical burden associated with BV (9) . The analysis of complex bacterial communities is hindered by conventional culture-dependent methods and by biochemical identification methods, as they leave many microorganisms uncultured and unidentified (9). The present molecular methods make it possible to differentiate between the closely related species of bacteria (10). Detailed information of complex microbial community can also be acquired from the phylogenetic analysis of 16S rDNA sequences obtained directly from samples by PCR amplification, cloning and sequencing so that unculturable species are also included (11).

Very little is known about the genetics of *G. vaginalis*, its physiology and the diversity of strains isolated from BV intermediate and BV positive women. Recent data has shown that the pathogenic strength or potential of *G. vaginalis* may be underestimated (12). To date, there have been no studies in our setting that have investigated the diversity of *G. vaginalis* detected in pregnant women. In this study, we aimed to investigate the diversity of the 16S ribosomal RNA (rRNA) of *G. vaginalis* detected in pregnant

women diagnosed as BV positive and intermediate. The findings of this study will add to the body of knowledge regarding the diversity of *G. vaginalis* across different BV states.

# CHAPTER 2

# 2.1 LITERATURE REVIEW

## 2.1.1 Overview

Bacterial vaginosis (BV) is the most common condition that affects women of child-bearing age (1). It is a condition whereby predominant *Lactobacillus* spp. in the healthy vagina, are replaced by a mixed flora including *Prevotella bivia, Prevotella disiens, Porphyromonas* spp. *Mobiluncus* spp. *and Peptostreptococcus* spp (2). Recent studies have shown an increased risk associated with multiple sexual partners (13). This condition BV, has been associated with adverse pregnancy outcomes, including premature rupture of membranes, chorioamnionitis, postpartum endometritis; and urinary tract infections (12-14). The mechanisms by which the BV-associated flora causes the signs of BV are not well understood, but the role for $H_2O_2$ producing *Lactobacillus* spp. in protecting against colonization by catalase-negative anaerobic bacteria has been reported (3).

## 2.1.2 History

*Gardnerella vaginalis* was originally discovered by Leopold (15) who described this microorganism as a "*Haemophilus*-like" species associated with prostatis and cervicitis (16). Gardner and Dukes (17) were the first to describe the microorganism in relation to bacterial vaginosis (BV). Due to its morphology, its apparent negative reaction to Gram staining and inability to grow on agar media lacking blood, researchers were convinced that they were dealing with a new *Haemophilus* species. They therefore named it based on its origin *Haemophilus vaginalis* (18)After extensive research it eventually became apparent that, unlike other members of the *Haemophilus* genus, '*H. vaginalis'* infrequently had a positive reaction to gram staining and did not require either hemin nor NAD for its growth. The microorganism was then temporarily placed into the *Corynebacterium* genus, and was referred to as *Corynebacterium vaginale* for quite some time (18, 19). However, since the bacterium was catalase negative and it lacked arabinose in its cell wall (17), it therefore did not fit into the description of the

*Corynebacterium* genus. Finally, two large taxonomic studies evaluating multiple criteria revealed the lack of similarities between "*H. vaginalis*" and other established genera (17, 20), a new genus named *Gardnerella* was proposed, with *G. vaginalis* being the only species in it.

### 2.1.3 Morphology

The cells of *G. vaginalis* are small, pleomorphic rods with average dimensions of 0.4 by 1.0–1.5 µm (15, 17), however, the length of some cells may reach up to 2–3 µm (20, 21). The cell size and morphology largely depend on their growth conditions and on their physiological state (17, 22). This bacterium is immotile, with the cells frequently occurring in clumps in vaginal smears and when grown in liquid media (20, 21). *G. vaginalis*, has a gram-positive cell wall (23), however due to its thin cell wall its gram staining varies from negative to positive it is therefore described as a Gram variable microorganism (16). *G. vaginalis* has fimbriae (pili) which are 3 to 7.5 nm in diameter covering the cell surface (24). The outer fibrillar coat is responsible for the attachment of *G. vaginalis* to the exfoliated vaginal epithelial cells also known as clue cells (24). Both fimbriae and exopolysaccharides are thought to be involved in attachment of *G. vaginalis* to the vaginal epithelium *in vivo* (16, 24). The cells of *G. vaginalis* are non-sporulating, they lack flagella, and they do not possess a typical capsule (20). *G. vaginalis* possess pili with a diameter 3 to 7.5nm. The presence of pili is more frequent in clinically isolated compared to laboratory cultured strains (25).

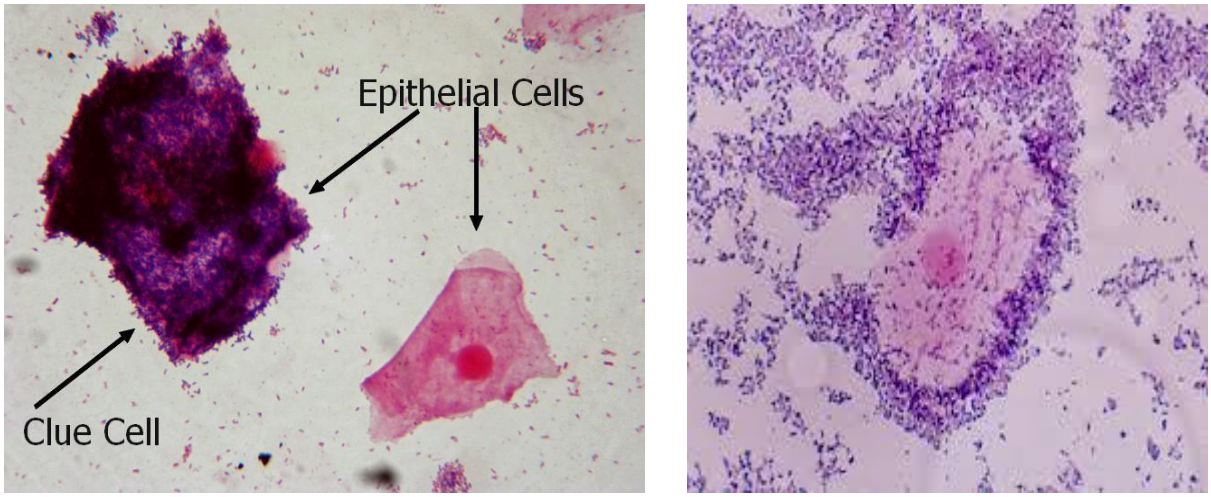


**Figure 1: A: *G. vaginalis* attached to exfoliated vaginal epithelial cells (clue cells). B: *G. vaginalis* as seen under microscopy after gram staining . (Centres for Disease Control and Prevention, 2010)**

### 2.1.4 Growth requirements, nutrition and metabolism

*Gardnerella vaginalis* is a facultative anaerobe and uses carbohydrates as its major energy source (20). This microorganism has a fermentative metabolism and acetic acid is a major product (26). *G. vaginalis* grows well in microaerophilic conditions in 5-7% $CO_2$. Optimum growth of *G. vaginalis* occurs at temperatures between 35 and $37^0$C, however, growth can occur between 25- $45^0$C . Optimum growth occurs at a pH between 6 and 6.5, no growth occurs at pH 4 and slight growth occurs at pH 4.5 and 8 (20). This micro-organism is fastidious, but it does not require either hemin or Nicotinamide Adenine Dinucleotide (NAD) for growth. As revealed by biochemical tests *G. vaginalis* is catalase-, oxidase- and β-glucosidase negative. It can ferment starch, dextrin, sucrose, glucose, fructose, ribose, maltose and raffinose. In addition, some strains of *G. vaginalis* can also ferment xylose and trehalose. However, *G. vaginalis* is unable to ferment rhamnose, melibiose, mannitol, and sorbitol (6). Furthermore, *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. Iron plays an important role in the growth of most pathogens (27). Jarosik *et al* 1998, studied the ability of *G. vaginalis* strains to acquire iron from the medium. Interestingly, all the strains they studied were able to acquire iron from ferric and ferrous inorganic substrates as well as hemin, catalase, haemoglobin, lactoferrin but not transferrin (27). Studies that analysed the genome of *G. vaginalis* revealed that the microorganism lacks enzymes in biochemical pathways that are involved in amino acid synthesis. It was predicted that *G. vaginalis* can synthesize some but not all purines and pyrimidine bases (6).

## 2.2 Transmission and Pathogenesis

*Gardnerella vaginalis* as a causative agent of BV adheres to host cell, multiplies and produce biofilms (28). In 2014 Schwebeke *et al* reported that *G. vaginalis* is transmitted through sexually activity with an infected partner (29). *G. vaginalis* has necessary virulence factors to adhere to host vaginal epithelium to compete with normal vaginal flora for dominance. In another study, they suggested that it is possible that *G. vaginalis*, lowers the reduction-oxidation potential in the vaginal microbiome through its metabolic pathways and ability to form biofilm (30). Biofilm is essential for the survival of BV associated pathogens in the vagina, enhancing a scaffold environment for the growth of anaerobes such as *Atopobium vaginae* and *Prevotella* spp. (31)**.** Biofilm formation has been reported to be the key for

the development of this condition as it provides elevated antibiotic tolerance and resistance to host immune defenses thereby resulting in the recurrent nature of this syndrome (7)**.** Sialidase which is produced by other strains of *G. vaginalis*, may enhance production of biofilm through its mucinase activity (32). The neurominidase sialidase produced by some strains of *G. vaginalis* interferes with host immune modulation resulting in adverse pregnancy outcomes (32). Sialidase activity in *G. vaginalis* was first described in 1984 (33). Sialidase also known as neuraminidases is a known virulence factor as it can invade and destroy tissue (34).

Vaginolysin is an important pore forming toxin, that can lyse human red blood cells and the vaginal epithelium resulting in tissue damage (35). In the case of BV, it has been shown that certain species of *Lactobacillus* produce inhibitory compounds or bacteriocins which act against *G. vaginalis* (29). It has also been shown *in vitro* that *G. vaginalis* produces bacteriocins that are also active against *Lactobacillus* (36).

## 2.3 Genetic diversity of *Gardnerella vaginalis*

Differences in virulence factors among the strains of *G. vaginalis* has been reported using genome sequencing (37). In 2010, a study examined the virulence factors for a strain of *G. vaginalis* from individuals diagnosed as BV positive and negative (6). The results of this study were interesting, as they found impaired adherence in epithelial cells in the non-BV isolate and suggested that there may be both commensal and pathogenic strains of *G. vaginalis*. However, as in other studies, they did not mention any Gram stain characteristics of the woman without BV. Recently, a comparative analysis of 17 clinical isolates of *G. vaginalis* suggested that the species can be subdivided into 4 clades and that there may be multiple species of *G. vaginalis*. Ahmed *et al* 2012 found that the degree of diversity among the strains was exceptionally high for a single species (37).

Studies based on 16S rRNA variable region targeted sequencing have been biased against detecting *Gardnerella* (38, 39) , however, one sequencing study distinguished four *G. vaginalis* subgroups based on a single nucleotide difference in variable region of the 16S rRNA gene (40). Santiago et al (32) studied 16S rRNA PCR product using *Taq* I restriction digest and they found that *G. vaginalis* has atleast three genotypes.

## 2.4 Epidemiology of Bacterial vaginosis (BV)

The prevalence of BV varies from country to country within the same region and within similar population groups. The prevalence has been estimated to be in the range 8% - 75% (42). The prevalence of BV has been shown to vary between ethnic groups in North America, South America, Europe, the Middle East and Asia. Based on a nationally representative sample of women in the United States, the prevalence has been estimated to be 21.2 million (29.2%) among women aged 14-49 years old (NHANES 2001-2004). The highest prevalence is seen in Africa and lowest in Asia and Europe (9).

In a recent study in South Africa, women infected with prevalent Herpes Simplex virus-2 (HSV-2) were shown to be at high risk for incident BV infections (41). This study investigated the risk of BV infections in HIV negative women, however, in a more recent study the prevalence of HSV-2 and HIV co-infection was reported to be 41% in South African women (42). Epidemiological data have indicated that the risk of BV is increased in women of African ethnicity (43). The possible risk factors of BV include population prevalence, socioeconomic and racial characteristics and other behavioural or physical risk factors such as smoking, the presence of sexually transmitted infections and underlying immune deficiencies such as those associated with HIV infection (44).

Recent epidemiological studies have sequenced bacterial communities present in BV using the molecular approach. According to the 5 to 8 bacterial communities present in the vaginal microbiome the data suggested that women with BV are prone to harbouring a specific cluster of microorganisms, while women at higher risk due to other established risk factors may harbour other BV-associated clusters as well (9). It was further hypothesized that biofilm-producing communities of *G. vaginalis* may play a role in the initiation and perpetuation of symptoms (9). The incidence of *G. vaginalis* has been shown to be significantly higher among women with preterm labour and late miscarriage (45).

## 2.5 Bacterial vaginosis during pregnancy

Bacterial vaginosis has been associated with preterm birth and poor perinatal outcomes (13). The vaginal microbiome in pregnancy plays an important role in both maternal and neonatal health outcomes (13, 14). A shift in the bacterial community of the vagina is observed during pregnancy to a composition that is dominated by one or two species of *Lactobacillus*. *Lactobacillus* is believed to inhibit the growth of pathogens through secretion of antibacterial bacteriocins as well as the production of metabolites such as lactic acid that help to maintain a low pH of 4.5 (46). Dysbiosis of the vaginal microbiome is

associated with complications of pregnancy, an increased risk of preterm birth (47). The maternal vaginal microbiome may also be an important source of pioneer bacteria for the neonatal gut microbiome (47), which have a profound effect on host system metabolism and immunity.

## 2.6 Diagnosis of BV

The diagnosis of BV has been problematic due to its complex polymicrobial nature (48). These include laboratory-based methods and clinical bedside testing.

### 2.6.1 Laboratory based method

Laboratory testing is based on microscopy of gram stained vaginal smears on a glass slide and air dried for gram staining by standard methods. The stained slide is examined under oil immersion and several bacterial morphotypes are evaluated by a standardized scoring method. Each slide is then graded as per the standardized quantitative morphological classification method developed by Spiegel et al (49) and later modified by Nugent et al (50), which assigns a score between 0 and 10 based on the abundance of the following various bacterial morphotypes: large Gram-positive rods (*Lactobacillus* morphotypes), small Gram-variable rods (*G. vaginalis* morphotypes), small Gram-negative rods (*Bacteroides* spp. morphotypes), curved Gram-variable rods (*Mobiluncus* spp. morphotypes), and Gram-positive cocci. Each morphotype is quantitated from 1 to 4+ regarding the number of morphotypes per oil immersion field (0, no morphotypes; 1+, less than 1 morphotype; 2+, 1 to 4 morphotypes; 3+, 5 to 30 morphotypes; and 4+, 30 or more morphotypes). Scores between 0 and 3 represent "normal vaginal flora," scores between 4 and 6 represent "intermediate vaginal flora," and scores between 7 and 10 are considered positive for BV. Nugent scoring is considered the standard method for BV diagnosis in research studies (50). Although highly accurate, this technique requires specialized training and is subjective, and microscopic evaluation is not widely available for clinicians to assist in the diagnosis of BV.

### 2.6.2 Molecular based methods

Applications of molecular methods to studies of the vaginal microbiome have not been limited to studies of microbially diverse populations and community composition alone but have also been applied as part of the diagnostic process for BV (43). Molecular techniques are well known for their high performance as they detect specific bacterial nucleic acids. Molecular techniques offer a wide range of advantages over microscopic techniques as they can detect fastidious bacteria, enable quantitation and most importantly they are highly sensitive when it comes to self-collected vaginal swabs. Several studies

have evaluated PCR assays for BV diagnosis (51-53) and obtained excellent results. Direct probe assays present a DNA probe to a vaginal fluid sample. The DNA probe binds specific sequences from a bacterium and can detect the presence of different bacteria in a single sample.

### 2.6.3 BD Max vaginal panel

The BD MAX vaginal panel is the quantitative real-time multiplex PCR assay (Becton Dickinson, Sparks, MD) that recently received FDA market authorization for the diagnosis of vaginitis in symptomatic women. The assay provides a positive or negative result for BV, *Candida* group (*C. albicans, C. tropicalis, C. parapsilosis, and C. dubliniensis*), *C. glabrata* or *C. krusei*, and *Trichomonas vaginalis* (54). It incorporates an automated DNA extraction and real time polymerase reaction (PCR) for the direct, qualitative detection of DNA from vaginal pathogens. This multiplex assay uses a proprietary algorithm to diagnose BV that includes a quantitative assessment of *Lactobacilli* (*L. crispatus* and *L. jensenii*), *G. vaginalis, A. vaginae*, *Megasphaera* type 1, and BV associated bacteria (BVAB2) (54).

### 2.6.4 Clinical methods

Clinically, the criteria that is mostly used is the Amsel's criteria, which requires at-least three of the following criteria to be met. The presence of clue cells constituting at-least 20% of vaginal epithelial cells in the vaginal fluid, an elevated vaginal pH of greater than 4.5, a release of a fishy (amine) odor upon addition of 10% potassium hydroxide to the vaginal fluid (48) and the presence of homogenous, non-viscous milky discharge adherent to vaginal walls . The production of amines results in an increased pH and favors the growth of other anaerobes that are associated with BV. Diagnosis based upon clinical signs without laboratory testing has 90% sensitivity but only 77% specificity (55).

## 2.7 Rationale for the proposed study

Currently there is a lack of published data on the genetic diversity of the 16S ribosomal RNA (rRNA) and *sialidase A* gene from *G. vaginalis* strains isolated from BV intermediate and positive pregnant women. This study is novel since it will be the first to explore the genetic diversity of positive and

intermediate *G. vaginalis* strains in South African pregnant women. By conducting this study, we would be able to shed light on the diversity of the strains of *G. vaginalis* in this population. Additionally, this would also give us an idea on therapeutic interventions as to whether the intermediate group should also be considered for treatment since the treatment focuses more on BV positive women.

## 2.8 Study objectives

2.8.1 To perform Nugent scoring on vaginal swabs to select BV intermediate and BV positive samples

2.8.2 To amplify the 16S rRNA gene of *G. vaginalis* from BV intermediate and BV positive samples by conventional PCR

2.8.3 To analyze the diversity in the 16S between positive and intermediate cases by sequencing and phylogenetic analyses

2.8.4 To detect and quantify the *sialidase A* gene of *G. vaginalis* by conventional PCR and droplet digital (ddPCR)

2.8.5 To analyze the diversity of the *sialidase A* gene between positive and intermediate cases by sequencing and phylogenetic analyses

# CHAPTER THREE

# METHODS

## 3.1 Ethics approval

The study was approved by the Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal (BE274/18).

## 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 n=354 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 the King Edward VIII hospital in Durban, South Africa. The women were classified as BV positive and intermediate 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, University of KwaZulu-Natal.

## 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 standardized quantitative morphological classification method developed by Nugent *et al* (50), 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 (Thermofischer Scientific, Invitrogen, Carlsbad, USA) according to manufacturer's instructions.

Briefly, 2 ml of DNA samples were centrifuged for 30 minutes at 14 000 *xg*. The supernatant was discarded and 800 µl of S1 lysis buffer was added to the pellet and pipetted up and down to mix the sample. The sample was then transferred to the bead tube and 100 µl of S2 lysis enhancer was added to the bead tube, capped and vortexed briefly. This was incubated at $95^0$C for 10 minutes, followed by vortexing at a maximum speed for 7 minutes and further centrifuged at 14 000 *xg* for 1 minute. Thereafter, 500 µl of the supernatant was transferred to a clean microcentrifuge tube, avoiding the bead pellet and any cell debris. To bind DNA to the column, 900 µl of binding buffer was added and vortexed briefly. Following this, 700 µl of the sample mixture was loaded onto a spin column-tube and centrifuged at 14 000 *xg* for 1 minute. The flow through was discarded and the spin column was centrifuged at 14 000 *xg* for 30 seconds. The spin column was placed in a clean tube, 50 µl of S6 elution buffer was added, the tube was incubated at room temperature for 1 minute. After 1 minute, the spin column was centrifuged at 14 000 *xg* for 1 minute, and the column was discarded and the purified DNA was stored at $-20^0$C.

### 3.3.3 PCR amplification of the 16S ribosomal region of *Gardnerella vaginalis*

To confirm the presence of *G. vaginalis* DNA in the extracted sample, a bacterium specific 16S ribosomal RNA (rRNA) PCR was performed for *G. vaginalis.* The PCR primers and cycling conditions used were as per (55). The following forward (5'GGGCGGGCTAGAGTGCA-3') and reverse primer pairs (5'GAACCCGTGGAATGGGCC-3') were used in the amplification reactions. The PCR conditions were as follows: an initial denaturation at 95ºC for 10 minutes followed by 40 cycles of 95ºC for 30 seconds (denaturation), annealing was performed at 55C for 30 seconds and extension at 72ºC for 30 seconds, followed by a final extension at 72ºC for 7 minutes. All PCR reactions were performed using a T100 thermocycler (BioRad). The PCR products were separated on a 1% agarose gel and viewed under a UV transilluminator (GENE GENIUS).

### 3.3.4 Detection of the *sialidase A* gene by PCR

The presence of the *sialidase A* gene was detected using the following specific primers; forward GVAGSIAF (GACGACGGCGAATGGCACGA) and reverse GVAGSIAR (TACAAGCGGCTTTACTCTTG). The PCR conditions were as follows: an initial denaturation at $95^0$C for 10 minutes followed by 40 cycles of denaturation at $95^0$C for 30 seconds, annealing was performed at $58^0$C for 1 minute and extension at $72^0$C for 2 minutes, this was followed by final extension at $72^0$C for 7 minutes. All PCR reactions were performed using a T100 thermocycler (BioRad). PCR products were separated on a 1% agarose gel and viewed under a UV transilluminator (GENE GENIUS).

### 3.3.5 Sequencing of 16S rRNA and *sialidase A* PCR amplicons

The PCR amplicons were sequenced using the Sanger method at Inqaba Biotechnological Industries in Pretoria, South Africa. The amplicons were sequenced using an ABI3500XL genetic analyser. The raw sequencing data was edited using Chromas software V2.6.5 (Technelysium.com).

### 3.3.6 Quantification of *sialidase A* gene by droplet digital PCR (ddPCR)

A total of 2.5ul of extracted DNA was used in the 20ul ddPCR reaction with the 2x digital PCR EvaGreen supermix. Primers specific to the *sialidase A* gene were added to the PCR mix (forward primer (GACGACGGCGAATGGCACGA); reverse primer (TACAAGCGGCTTTACTCTTG).
Droplets were generated using the manual droplet generator (Bio-Rad), droplet generation oil for EvaGreen (Bio-Rad) and the PCR mix containing the sample. A total of 40ul of droplets were used for the PCR reaction, with the following conditions; 95℃ for 10 min, 40 cycles of 94℃ for 30 sec and 60℃ for 1 min, and 98℃ for 10 min. Samples were read on the QX200 Droplet Reader (Bio-Rad) using the QuantaSoft Software and acquired on Ch1 for FAM. Analysis was performed on the QuantaSoft Software using manual thresholding. Statistical analysis was performed on GraphPad Prism 5.

## 3.4 Phylogenetic analysis

Phylogenetic trees were constructed using the neighbour-joining (NJ) method based on nucleotide distances calculated by Kimura's 2-parameter method across relevant segments of the alignment. Gaps were excluded on a pairwise basis. The NJ tree generated was the result of 1000 bootstrap replications from a ClustalW multiple alignment. The PHYLogeny Inference Package (PHYLIP) was used for the phylogenetic analysis.

### 3.5 Data analyses

Statistical analyses, including means values, standard deviations, standard errors, p values and 95% confidence intervals (CI) was performed using Graph Pad Prism 5.

# CHAPTER 4

# RESULTS

## 4. Demographic results

### 4.1 Characteristics of the study population

Table 1 describes the characteristics of the study population. Within the BV intermediate group, a higher proportion of the women were between the age of 2529 years old (41.18%). A higher proportion of women in this group did not complain of abnormal vaginal discharge (67.65%). Most women who had been diagnosed as BV intermediate were unmarried (91.18%), had completed high school (79.41%), had a regular sexual partner (79.41%), had experience first sex at 15-20 years of age (82.35%), had between 2-4 life time sex partners (47.06%) and did not practise condom use at their last sex act (82.35%) respectively. In addition, most women who were intermediate were in the third trimester of pregnancy (58.82%).

For the women who were diagnosed as BV positive, similarly to the intermediate group, most women who were between the ages of 25-29 years (33.33%), did not complain of abnormal discharge (64.81%) had completed high school (64.81%) and were unmarried (92.59%). A higher proportion of BV positive women were also in the third trimester of pregnancy (51.85%), did not use condoms(62.96%). and has 2-4 life-time sex partners (48.15%).

**Table 1: Characteristics of the study population**

| | BV Intermediate | | BV Positive | | Total | |
|---|---|---|---|---|---|---|
| | **N** | **Percentage** | **N** | **Percentage** | **N** | **Percentage** |
| **Number sampled** | **34** | **38.6** | **54** | **61.4** | **88** | **100** |
| **Age group** | | | | | | |
| 15-19 | 1 | 2.94 | 2 | 3.7 | 3 | 3.41 |
| 20-24 | 9 | 26.47 | 10 | 18.52 | 19 | 21.59 |
| 25-29 | 14 | 41.18 | 18 | 33.33 | 32 | 36.36 |
| 30-34 | 6 | 17.65 | 13 | 24.07 | 19 | 21.59 |
| 35-39 | 3 | 8.82 | 9 | 16.67 | 12 | 13.64 |
| 40-44 | 1 | 2.94 | 2 | 3.7 | 3 | 3.41 |
| **Current abnormal vaginal discharge** | | | | | | |
| No | 23 | 67.65 | 35 | 64.81 | 58 | 65.91 |
| Yes | 11 | 32.35 | 19 | 35.19 | 30 | 34.09 |
| **Educational level** | | | | | | |
| College/University | 7 | 20.59 | 15 | 27.78 | 22 | 25.00 |
| High school | 27 | 79.41 | 35 | 64.81 | 62 | 70.45 |
| Primary school | 0 | 0 | 4 | 7.41 | 4 | 4.55 |
| **Married** | | | | | | |
| No | 31 | 91.18 | 50 | 92.59 | 81 | 92.05 |
| Yes | 3 | 8.82 | 4 | 7.41 | 7 | 7.95 |
| **Regular sex partner** | | | | | | |
| No | 7 | 20.59 | 4 | 7.41 | 11 | 12.50 |
| Yes | 27 | 79.41 | 50 | 92.59 | 77 | 87.50 |
| **Cohabiting** | | | | | | |
| No | 19 | 55.88 | 34 | 62.96 | 53 | 60.23 |
| Yes | 15 | 44.12 | 20 | 37.04 | 35 | 39.77 |
| **Age of first sex** | | | | | | |
| 15-20 | 28 | 82.35 | 42 | 77.78 | 70 | 79.55 |
| 21-25 | 5 | 14.71 | 7 | 2.96 | 12 | 3.64 |
| <15 | 0 | 0 | 4 | 7.41 | 4 | 4.55 |
| >25 | 1 | 2.94 | 1 | 1.85 | 2 | 2.27 |
| **No of lifetime sex partners** | | | | | | |
| 1 | 11 | 32.35 | 14 | 25.93 | 25 | 28.41 |
| 2-4 | 16 | 47.06 | 26 | 48.15 | 42 | 47.73 |
| >4 | 7 | 20.59 | 14 | 25.93 | 21 | 23.86 |
| **Partner has other partners** | | | | | | |
| Don’t know | 14 | 41.18 | 19 | 35.19 | 33 | 37.5 |
| No | 11 | 32.35 | 14 | 25.93 | 25 | 28.41 |
| Yes | 9 | 26.47 | 21 | 38.89 | 30 | 34.09 |
| **Condom used during last sex act** | | | | | | |
| No | 28 | 82.35 | 34 | 62.96 | 62 | 70.45 |
| Yes | 6 | 7.65 | 20 | 37.04 | 26 | 29.55 |

| Trimester pregnancy | | | | | | |
|---|---|---|---|---|---|---|
| 1st | 6 | 17.65 | 9 | 16.67 | 15 | 17.05 |
| 2nd | 8 | 23.53 | 17 | 31.48 | 25 | 28.41 |
| 3rd | 20 | 58.82 | 28 | 51.85 | 48 | 54.55 |

## 4.2 LABORATORY RESULTS

### 4.2.1 Diagnosis of BV based on Nugent scoring

To classify pregnant women as either BV positive or BV intermediate, vaginal smears were assessed by the Nugent scoring criteria. Of the 354 characterized vaginal swab samples, a total of n=37 intermediate cases were identified in the study population. Although n=124 BV positive cases were identified, for this study, n=50 positive samples were randomly selected for further analysis.

### 4.2.2 Amplification of the 16S and *sialidase A* gene

The 16S rRNA was successfully amplified using the bacterium specific primers without the need to optimize the PCR. All BV positive and BV intermediates samples produced positive amplicons (Fig. 2). The *sialidase A* gene was shown to be present in 47/50 (95%) of the BV positive samples and 30/37 (81%) of the intermediate samples (Fig. 3).

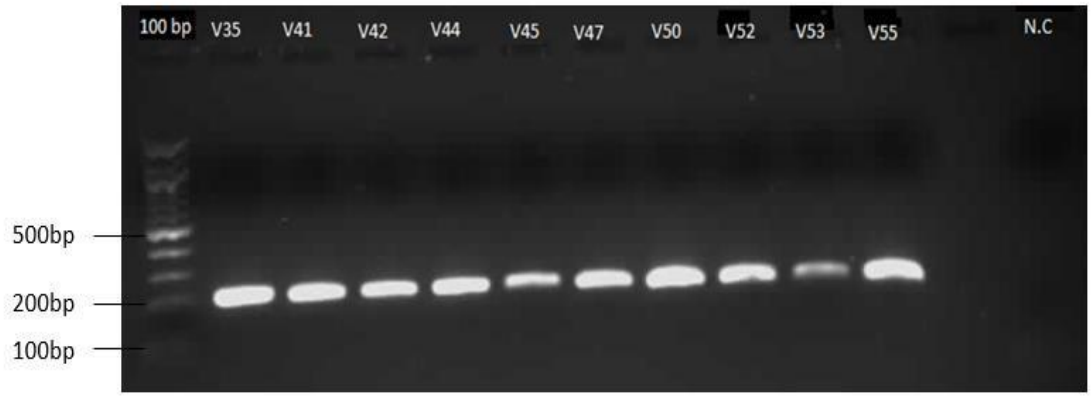


**Figure 2: Agarose gel electrophoresis of selected BV positive samples (n=10). Lane 1: 100 bp molecular weight marker (Fermentas), Lane 2 – lane 10 (positive bacterial vaginosis samples), and Lane 12 Negative control (N.C).**

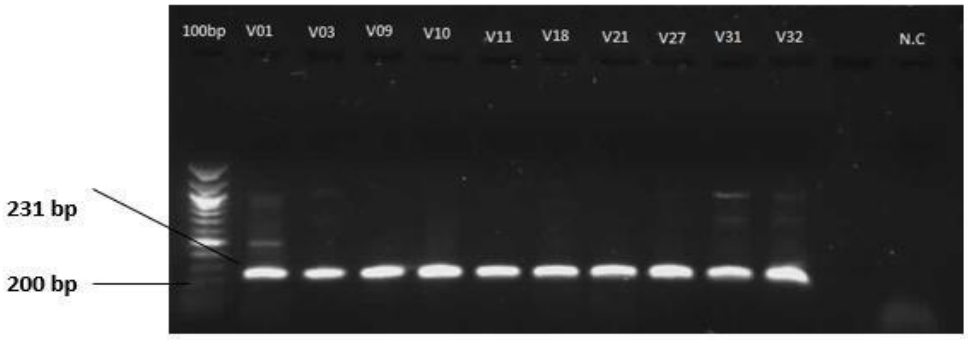


**Figure 3: Agarose gel electrophoresis of selected BV positive samples (n=10) to confirm the presence of the *sialidase A* gene. Lane 1:100bp molecular weight marker (Fermentas), Lane 2-11: BV samples, and Lane 13: Negative control.**

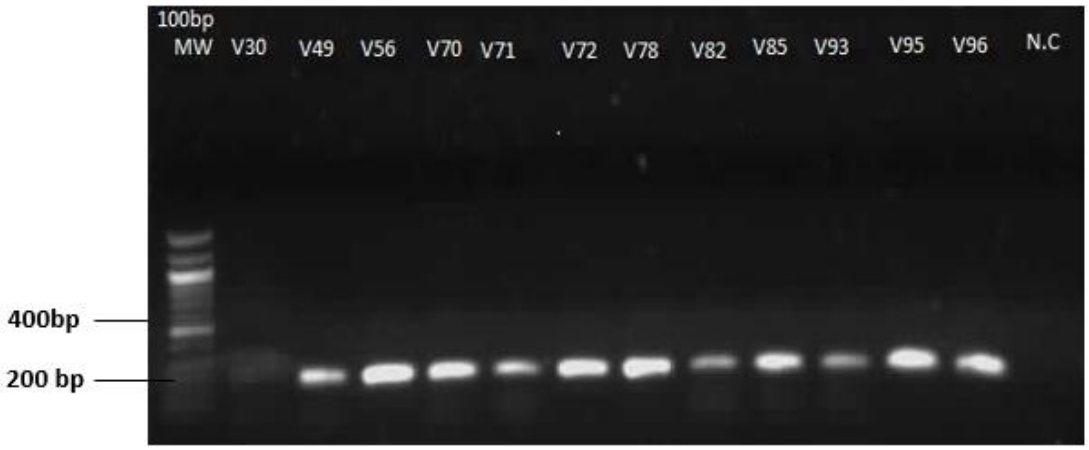


**Figure 4: Agarose gel electrophoresis of the *sialidase A* gene of selected BV intermediate samples (n=11).**

### 4.3 Sequence alignment and Phylogenetic analysis of *G. vaginalis* 16S rRNA sequences

To determine the genetic differences in the 16S rRNA across BV positive and intermediates cases, a phylogenetic analysis was performed. According to the sequence alignment of all samples analyzed, there were no genetic differences in the sequences across the samples (Appendix 1). This was also shown in the neighbor joining phylogenetic tree which was constructed (Figure 5). The sequences were aligned to study the genetic differences within the 16S rRNA of the *G. vaginalis* PCR amplicons. The sequence alignment was performed using Bioedit.

Table 2 shows the Basic Local Alignment Search Tool (BLAST) search results for all 16S rRNA amplicons that were generated. For both BV positive and intermediates the percent identity to the *G. vaginalis* ribosomal RNA ranged from 90 -100%.

**Table 2: Basic Local Alignment Search tool (BLAST) results for 16S rRNA amplicons of *G. vaginalis*.**

| Sample no. | Result | % Identity | Accession number |
|---|---|---|---|
| V01 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 99.28 | gi\|1530815055\|MH898666.1 |
| V03 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 99.36 | gi\|1530815055\|MH898666.1 |
| V09 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |
| V10 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |
| V11 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 99.39 | gi\|1530815055\|MH898666.1 |
| V13 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 98.52 | gi\|1530815055\|MH898666.1 |
| V17 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |
| V18 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |

| | | | |
|---|---|---|---|
| V19 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |
| V21 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |
| V24 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 80 | gi\|1530815055\|MH898666.1 |
| V27 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |
| V30 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 98.78 | gi\|1530815055\|MH898666.1 |
| V31 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 99.39 | gi\|1530815055\|MH898666.1 |
| V32 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |
| V35 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |
| V41 | G. vaginalis strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |
| V42 | G. vaginalis strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |
| V44 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |
| V45 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 98.37 | gi\|1530815055\|MH898666.1 |
| V47 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 99.36 | gi\|1530815055\|MH898666.1 |
| V49 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 99.39 | gi\|1530815055\|MH898666.1 |
| V50 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 99.37 | gi\|1530815055\|MH898666.1 |
| V52 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |
| V53 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |

| | | | |
|---|---|---|---|
| V55 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |
| V56 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |
| V57 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |
| V58 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |
| V60 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |
| V64 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |
| V66 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 99.40 | gi\|1530815055\|MH898666.1 |
| V67 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |
| V68 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 98.78 | gi\|1530815055\|MH898666.1 |
| V69 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |
| V70 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |
| V71 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 99.37 | gi\|1530815055\|MH898666.1 |
| V72 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 99.35 | gi\|1530815055\|MH898666.1 |
| V73 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |
| V74 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |
| V76 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |

| | | | |
|---|---|---|---|
| V78 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 99.37 | gi\|1530815055\|MH898666.1 |

| | | | |
|---|---|---|---|
| V81 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |
| V82 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 99.32 | gi\|1530815055\|MH898666.1 |
| V83 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |
| V84 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |
| V85 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 99.37 | gi\|1530815055\|MH898666.1 |
| V88 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |
| V92 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |
| V93 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 98.17 | gi\|1530815055\|MH898666.1 |
| V95 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |
| V96 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |
| V98 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 99.39 | gi\|1530815055\|MH898666.1 |
| V101 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 99.36 | gi\|1530815055\|MH898666.1 |
| V102 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 98.79 | gi\|1530815055\|MH898666.1 |
| V103 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |
| V109 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |

| | | | |
|---|---|---|---|
| V111 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |
| V112 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |

| | | | |
|---|---|---|---|
| V116 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |
| V117 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 98.78 | gi\|1530815055\|MH898666.1 |
| V121 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 99.36 | gi\|1530815055\|MH898666.1 |
| V123 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 99.36 | gi\|1530815055\|MH898666.1 |
| V124 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |
| V126 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 99.39 | gi\|1530815055\|MH898666.1 |
| V184 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 99.36 | gi\|1530815055\|MH898666.1 |
| V202 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |
| V206 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 98.58 | gi\|1530815055\|MH898666.1 |
| V207 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 99.36 | gi\|1530815055\|MH898666.1 |
| V239 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 99.39 | gi\|1530815055\|MH898666.1 |
| V240 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 98.78 | gi\|1530815055\|MH898666.1 |
| V250 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 99.36 | gi\|1530815055\|MH898666.1 |
| V258 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 98.78 | gi\|1530815055\|MH898666.1 |

| | | | |
|---|---|---|---|
| V269 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 99.39 | gi\|1530815055\|MH898666.1 |
| V270 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 99.36 | gi\|1530815055\|MH898666.1 |
| V276 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 98.61 | gi\|1530815055\|MH898666.1 |
| V277 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 98.78 | gi\|1530815055\|MH898666.1 |
| V281 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 100 | gi\|1530815055\|MH898666.1 |
| V292 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 99 | gi\|1530815055\|MH898666.1 |
| V312 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 99.79 | gi\|1530815055\|MH898666.1 |
| V319 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 99.37 | gi\|1530815055\|MH898666.1 |
| V320 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 95.65 | gi\|1530815055\|MH898666.1 |
| V324 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 98.79 | gi\|1530815055\|MH898666.1 |
| V328 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 98.73 | gi\|1530815055\|MH898666.1 |
| V343 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 99.37 | gi\|1530815055\|MH898666.1 |
| V350 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 98.78 | gi\|1530815055\|MH898666.1 |
| V353 | *G. vaginalis* strain UGent25.49 16S rRNA, partial sequence | 99.37 | gi\|1530815055\|MH898666.1 |

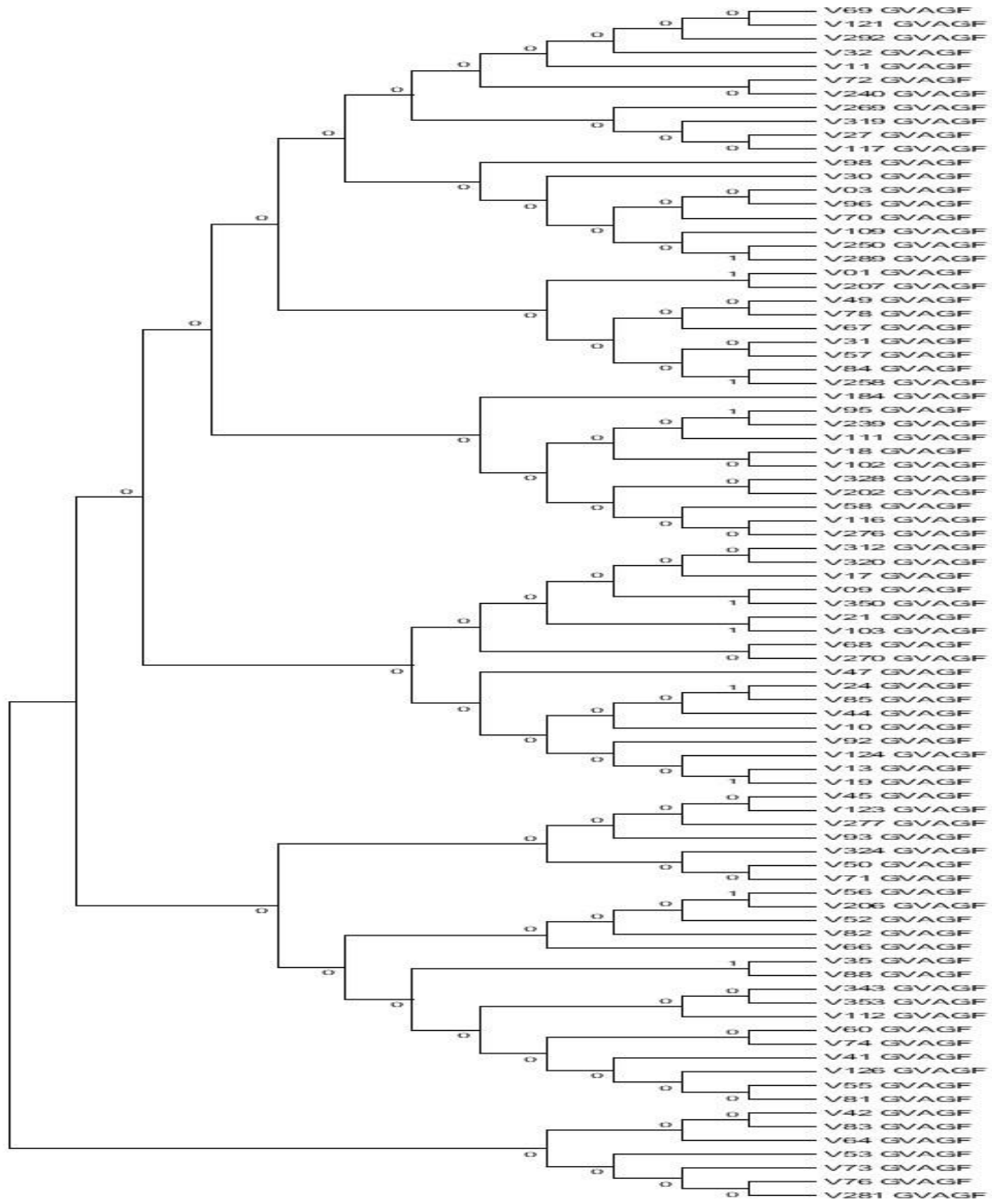


**Figure 5: The evolutionary history was inferred using the UPGMA method. The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths 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. Phylogenetic analyses were conducted in MEGA4.**

## 4.4 Quantification of *Sialidase A* gene by Droplet digital PCR

Droplet digital PCR enables absolute quantification of nucleic acids in a sample. In the present study, 77 DNA extracts were tested (47 BV positives and 30 BV intermediates). These were confirmed to contain the *sialidase A* gene by conventional PCR. EvaGreen Supermix for probes and droplet generation oil was used, forward and reverse primers were also used. A high number of *sialidase A* copies was observed in BV positive cases compared to BV intermediate cases. However, there are BV intermediate cases that had high copy numbers of *sialidase A* as well. With reference to Table 3, V32, V09, V10 BV positives had the highest number of copies of *sialidase A* 69000, 55600, and 46600 copies per 20ul of reactions, respectively. A high copy number of *sialidase A* was also observed in intermediate BV cases, with V116, V72, V124 having a high copy number of 45240, 40940 and 38900 copies per 20ul respectively.

**Table 3: Quantification of sialidase A in BV positive (n=47) and 30 BV intermediate (n=30) by ddPCR.**

| Sample | Concentration per ul | Copies per 20uL Well | Poisson Confidence Max | Poisson Confidence Min | Positive Droplets | Negative droplets | Accepted Droplets | Threshold | BV status |
|---|---|---|---|---|---|---|---|---|---|
| V01 | 12,9 | 258 | 14,7 | 11,1 | 197 | 17829 | 18026 | 10000 | Pos |
| V03 | 329 | 6580 | 339 | 320 | 4731 | 14641 | 19372 | 10000 | Pos |
| V09 | 2780 | 55600 | 2830 | 2730 | 15883 | 1652 | 17535 | 10000 | Pos |
| V10 | 2330 | 46600 | 2374 | 2288 | 15367 | 2460 | 17827 | 10000 | Pos |
| V11 | 891 | 17820 | 909 | 873 | 10128 | 8941 | 19069 | 10000 | Pos |
| V13 | 2,9 | 58 | 3,8 | 2,1 | 48 | 19541 | 19589 | 10000 | Pos |
| V17 | 9,8 | 196 | 11,3 | 8,3 | 159 | 18990 | 19149 | 10000 | Pos |
| V18 | 28 | 560 | 30,5 | 25,4 | 461 | 19152 | 19613 | 10000 | Pos |
| V19 | 444 | 8880 | 456 | 432 | 5469 | 11923 | 17392 | 10000 | Pos |
| V21 | 412 | 8240 | 424 | 401 | 4957 | 11815 | 16772 | 10000 | Pos |
| V24 | 3,9 | 78 | 5 | 3 | 56 | 16833 | 16889 | 10000 | Pos |
| V27 | 1217 | 24340 | 1241 | 1193 | 11073 | 6108 | 17181 | 10000 | Pos |
| V31 | 221 | 4420 | 229 | 213 | 2999 | 14533 | 17532 | 10000 | Pos |
| V32 | 3450 | 69000 | 3530 | 3370 | 14608 | 824 | 15432 | 10000 | Pos |
| V35 | 155 | 3100 | 162 | 149 | 2196 | 15562 | 17758 | 10000 | Pos |
| V42 | 1082 | 21640 | 1103 | 1061 | 10974 | 7275 | 18249 | 10000 | Pos |
| V44 | 3,7 | 74 | 4,7 | 2,9 | 64 | 20248 | 20312 | 10000 | Pos |
| V45 | 4 | 80 | 5 | 3,1 | 68 | 20006 | 20074 | 10000 | Pos |
| V47 | 92,4 | 1848 | 97 | 87,8 | 1552 | 19000 | 20552 | 10000 | Pos |
| V49 | 2,3 | 46 | 3,1 | 1,6 | 37 | 18932 | 18969 | 10000 | Pos |
| V50 | 886 | 17720 | 904 | 869 | 10000 | 8897 | 18897 | 10000 | Pos |
| V53 | 326 | 6520 | 336 | 316 | 4344 | 13615 | 17959 | 10000 | Pos |
| V55 | 73,6 | 1472 | 77,7 | 69,5 | 1212 | 18777 | 19989 | 10000 | Pos |
| V57 | 152 | 3040 | 158 | 146 | 2374 | 17248 | 19622 | 10000 | Pos |
| V58 | 26,1 | 522 | 28,6 | 23,6 | 404 | 18013 | 18417 | 10000 | Pos |
| V60 | 1865 | 37300 | 1900 | 1832 | 14336 | 3693 | 18029 | 10000 | Pos |
| V64 | 28,1 | 562 | 31,5 | 24,8 | 276 | 11398 | 11674 | 10000 | Pos |
| V67 | 144 | 2880 | 150 | 138 | 2171 | 16633 | 18804 | 10000 | Pos |
| V68 | 18,2 | 364 | 20,3 | 16,2 | 303 | 19396 | 19699 | 10000 | Pos |
| V69 | 1047 | 20940 | 1067 | 1027 | 11008 | 7672 | 18680 | 10000 | Pos |
| V73 | 5,9 | 118 | 7,2 | 4,8 | 98 | 19376 | 19474 | 10000 | Pos |
| V74 | 72,4 | 1448 | 76,4 | 68,3 | 1221 | 19242 | 20463 | 10000 | Pos |
| V76 | 94,3 | 1886 | 99,1 | 89,5 | 1478 | 17713 | 19191 | 10000 | Pos |
| V81 | 3530 | 70600 | 3610 | 3460 | 18200 | 951 | 19151 | 10000 | Pos |
| V83 | 434 | 8680 | 445 | 423 | 5868 | 13161 | 19029 | 10000 | Pos |
| V84 | 33,4 | 668 | 36,2 | 30,6 | 560 | 19450 | 20010 | 10000 | Pos |
| V88 | 162 | 3240 | 170 | 154 | 1637 | 11111 | 12748 | 10000 | Pos |
| V92 | 3890 | 77800 | 3980 | 3800 | 17190 | 654 | 17844 | 10000 | Pos |
| V98 | 75,4 | 1508 | 79,7 | 71,1 | 1171 | 17700 | 18871 | 10000 | Pos |
| V103 | 4,5 | 90 | 5,6 | 3,6 | 77 | 19907 | 19984 | 10000 | Pos |
| V109 | 4,9 | 98 | 6 | 3,9 | 78 | 18765 | 18843 | 10000 | Pos |
| V111 | 24,2 | 484 | 26,6 | 21,8 | 390 | 18774 | 19164 | 10000 | Pos |
| V112 | 126 | 2520 | 133 | 120 | 1463 | 12935 | 14398 | 10000 | Pos |
| V116 | 2262 | 45240 | 2304 | 2221 | 15219 | 2607 | 17826 | 10000 | Pos |
| V121 | 15 | 300 | 16,6 | 13,3 | 316 | 24690 | 25006 | 10000 | Pos |
| V123 | 14,4 | 288 | 16,2 | 12,6 | 246 | 19967 | 20213 | 10000 | Pos |
| V124 | 1945 | 38900 | 1980 | 1911 | 15496 | 3668 | 19164 | 10000 | Pos |
| V56 | 10,2 | 204 | 11,8 | 8,7 | 173 | 19804 | 19977 | 10000 | Int |
| V72 | 2047 | 40940 | 2083 | 2012 | 16115 | 3431 | 19546 | 10000 | Int |
| V78 | 1008 | 20160 | 1035 | 981 | 5603 | 4134 | 9737 | 10000 | Int |
| V85 | 1,4 | 28 | 2,1 | 0,9 | 24 | 19783 | 19807 | 10000 | Int |
| V93 | 1,8 | 36 | 2,5 | 1,2 | 28 | 18584 | 18612 | 10000 | Int |
| V95 | 1,5 | 30 | 2,2 | 1 | 25 | 19704 | 19729 | 10000 | Int |
| V102 | 2,3 | 46 | 3,1 | 1,6 | 37 | 18953 | 18990 | 10000 | Int |
| V117 | 3 | 60 | 3,9 | 2,2 | 48 | 18862 | 18910 | 10000 | Int |
| V126 | 700 | 14000 | 716 | 685 | 8213 | 10099 | 18312 | 10000 | Int |
| V184 | 2,1 | 42 | 2,9 | 1,4 | 32 | 18287 | 18319 | 10000 | Int |
| V202 | 697 | 13940 | 713 | 681 | 7682 | 9500 | 17182 | 10000 | Int |
| V206 | 51 | 1020 | 54,6 | 47,4 | 773 | 17436 | 18209 | 10000 | Int |
| V207 | 1095 | 21900 | 1117 | 1074 | 10504 | 6832 | 17336 | 10000 | Int |
| V250 | 300 | 6000 | 309 | 290 | 3879 | 13371 | 17250 | 10000 | Int |
| V269 | 5,7 | 114 | 7 | 4,6 | 88 | 17995 | 18083 | 10000 | Int |
| V270 | 1 | 20 | 1,7 | 0,6 | 13 | 14601 | 14614 | 10000 | Int |
| V276 | 0,63 | 12,6 | 1,12 | 0,32 | 10 | 18568 | 18578 | 10000 | Int |
| V277 | 14 | 280 | 15,8 | 12,2 | 228 | 19048 | 19276 | 10000 | Int |
| V281 | 6,5 | 130 | 7,9 | 5,3 | 95 | 17200 | 17295 | 10000 | Int |
| V289 | 12,3 | 246 | 14,1 | 10,6 | 189 | 17923 | 18112 | 10000 | Int |
| V292 | 1370 | 27400 | 1396 | 1345 | 12148 | 5508 | 17656 | 10000 | Int |
| V312 | 190 | 3800 | 199 | 181 | 1867 | 10649 | 12516 | 10000 | Int |
| V319 | 2 | 40 | 2,8 | 1,4 | 30 | 17801 | 17831 | 10000 | Int |
| V320 | 0,74 | 14,8 | 1,24 | 0,39 | 12 | 19109 | 19121 | 10000 | Int |
| V324 | 349 | 6980 | 359 | 339 | 4688 | 13590 | 18278 | 10000 | Int |
| V328 | 845 | 16900 | 866 | 823 | 6258 | 5960 | 12218 | 10000 | Int |
| V343 | 3,1 | 62 | 4,2 | 2,1 | 34 | 13006 | 13040 | 10000 | Int |
| V350 | 580 | 11600 | 594 | 567 | 7121 | 11169 | 18290 | 10000 | Int |
| V353 | 36,1 | 722 | 39,2 | 33,1 | 546 | 17511 | 18057 | 10000 | Int |

### 4.5 *Sialidase A* gene copy number in relation to BV status

Following ddPCR, *sialidase A* copy numbers in association with BV positives and BV intermediates was investigated (Fig. 6). In the BV positive group the mean value was 13374 (95% CI 7203-19545) and in the intermediate group the mean value was 5760 (95% CI 1941-9579). The mean value of BV positives in association with *sialidase A* was high (double the mean value of the BV intermediates). According to Fig.6 the average copies of *sialidase A* per cell is significantly much higher in the BV positive group when compared to the BV intermediate group.

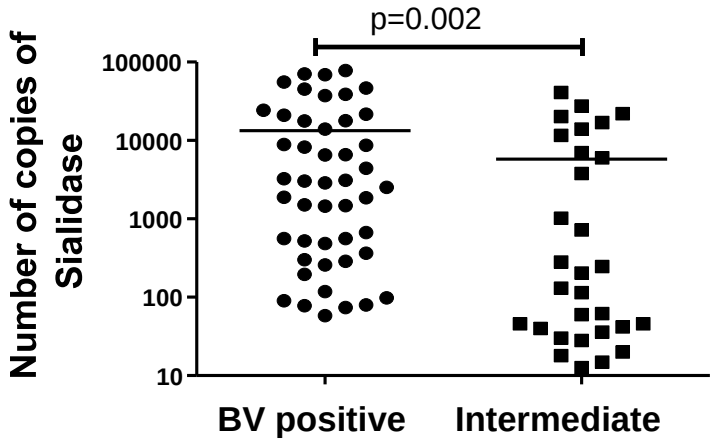


**Figure 6: *Sialidase A* copy numbers associated with the BV positive and BV intermediate groups, p=0.002.**

To further analyze these results, the number of copies of *sialidase A* in each sample was compared with the presence or absence of clinical symptoms of BV namely, abnormal vaginal discharge (Figure 7). Interestingly, of the 77 samples analyzed, 25 (32 %) of the participants had abnormal vaginal discharge and 52 (68 %) had no discharge. The mean value in the group with the presence of discharge was 14 536 (95% CI 6545- 22527) whereas with the group reporting no discharge the mean value was 8422 (95% CI 3681-13163); p= 0.003. According to Figure 7, there is a significant difference between the two groups. The high copy numbers of *sialidase A* gene was associated with the presence of discharge.

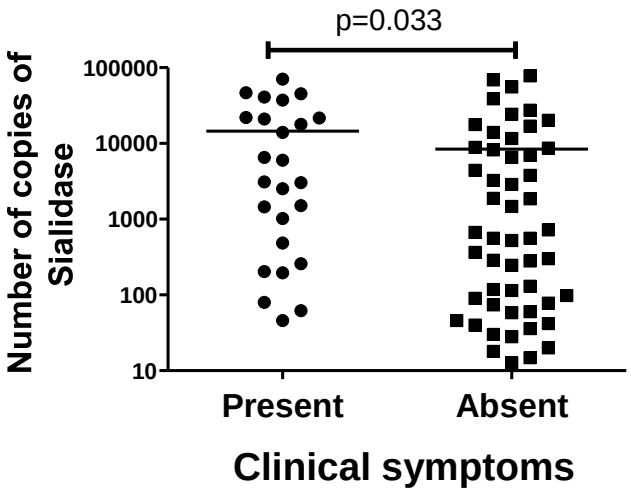


**Figure 7: Association of *sialidase A* copy numbers with the presence or absence of BV clinical symptoms (abnormal vaginal discharge); p= 0.033.**

## 4.6 Sequencing and phylogenetic analysis of sialidase A gene

A subset of 20 *sialidase A* amplicons were sequenced, BV positives (n=13) with high copy numbers of *sialidase A* and BV intermediates (n=7) with low copy numbers for *sialidase A*. However, after sequencing, 5 BV intermediate samples were available for the phylogenetic analysis since 2 samples did not sequence properly and were excluded from the analysis.

With reference to Table 4 which shows BLAST search results of *sialidase A* gene sequences, the percent identity ranged from 91- 100% for most samples with only V81 showing 100% identity to *G. vaginalis sialidase A* gene**.**

**Table 4: Basic Local Alignment Search tool (BLAST) results for *sialidase A* gene amplicons of *G. vaginalis.***

| Sample | Result | % identity | Accession no. |
|---|---|---|---|
| V01 | Gardnerella vaginalis strain 79.2 sialidase A gene, complete cds | 94.47 | gi\|1349730490\|MG737374.1 |
| V03 | Gardnerella vaginalis strain 60.1 sialidase A gene, complete cds | 92.93 | gi\|1349730488\|MG737373.1 |
| V10 | Gardnerella vaginalis strain 114.2 sialidase A gene, complete cds | 91.01 | gi\|1349730494\|MG737376.1 |
| V11 | Gardnerella vaginalis strain 60.1 sialidase A gene, complete cds | 97.97 | gi\|1349730488\|MG737373.1 |
| V17 | Gardnerella vaginalis strain 114.2 sialidase A gene, complete cds | 94.76 | gi\|1349730494\|MG737376.1 |
| V19 | Gardnerella vaginalis strain 58.4 sialidase A gene, complete cds | 94.18 | gi\|1349730486\|MG737372.1 |
| V35 | Gardnerella vaginalis strain 86.1 sialidase A gene, complete cds | 91.96 | gi\|1349730492\|MG737375.1 |
| V42 | Gardnerella vaginalis strain 60.1 sialidase A gene, complete cds | 90.43 | gi\|1349730488\|MG737373.1 |
| V73 | Gardnerella vaginalis strain 60.1 sialidase A gene, complete cds | 76.53 | gi\|1349730488\|MG737373.1 |
| V81 | Gardnerella vaginalis strain 58.4 sialidase A gene, complete cds | 100 | gi\|1349730486\|MG737372.1 |
| V116 | Gardnerella vaginalis strain 58.4 sialidase A gene, complete cds | 94.15 | gi\|1349730486\|MG737372.1 |
| V121 | Gardnerella vaginalis strain 60.1 sialidase A gene, complete cds | 89.50 | gi\|1349730488\|MG737373.1 |
| V123 | Gardnerella vaginalis strain 60.1 sialidase A gene, complete cds | 91.62 | gi\|1349730488\|MG737373.1 |

| | | | |
|---|---|---|---|
| V184 | Gardnerella vaginalis strain 60.1 sialidase A gene, complete cds | 92.22 | gi\|1349730488\|MG737373.1 |
| V202 | Gardnerella vaginalis strain 60.1 sialidase A gene, complete cds | 95.19 | gi\|1349730488\|MG737373.1 |
| V207 | Gardnerella vaginalis strain 60.1 sialidase A gene, complete cds | 96.48 | gi\|1349730488\|MG737373.1 |
| V277 | Gardnerella vaginalis strain 60.1 sialidase A gene, complete cds | 95.16 | gi\|1349730488\|MG737373.1 |
| V281 | Gardnerella vaginalis strain 60.1 sialidase A gene, complete cds | 95.96 | gi\|1349730488\|MG737373.1 |

The sequences were aligned to study the genetic differences between the *sialidase A* gene from BV positive and intermediate cases. Appendix 2 shows the sequence alignment of 13 BV positive and 5 BV intermediate sequences. Comparing the alignment and the phylogenetic tree (Figure 8) the sequences that harbour the same mutations clustered together in the phylogenetic tree. Sample V17 (BV positive) and V184 (BV intermediate) clustered together in (Figure 8) and share a common mutation at position A38G in both sequences and at position A65C (Appendix 2). Samples V116, V123, V207, V277 and V 281 also harboured the same mutations at position 21 whereby G was replaced by Cytosine (C) and at position A38R. According to the phylogenetic tree, BV intermediate samples were distributed across the tree (Figure 8).

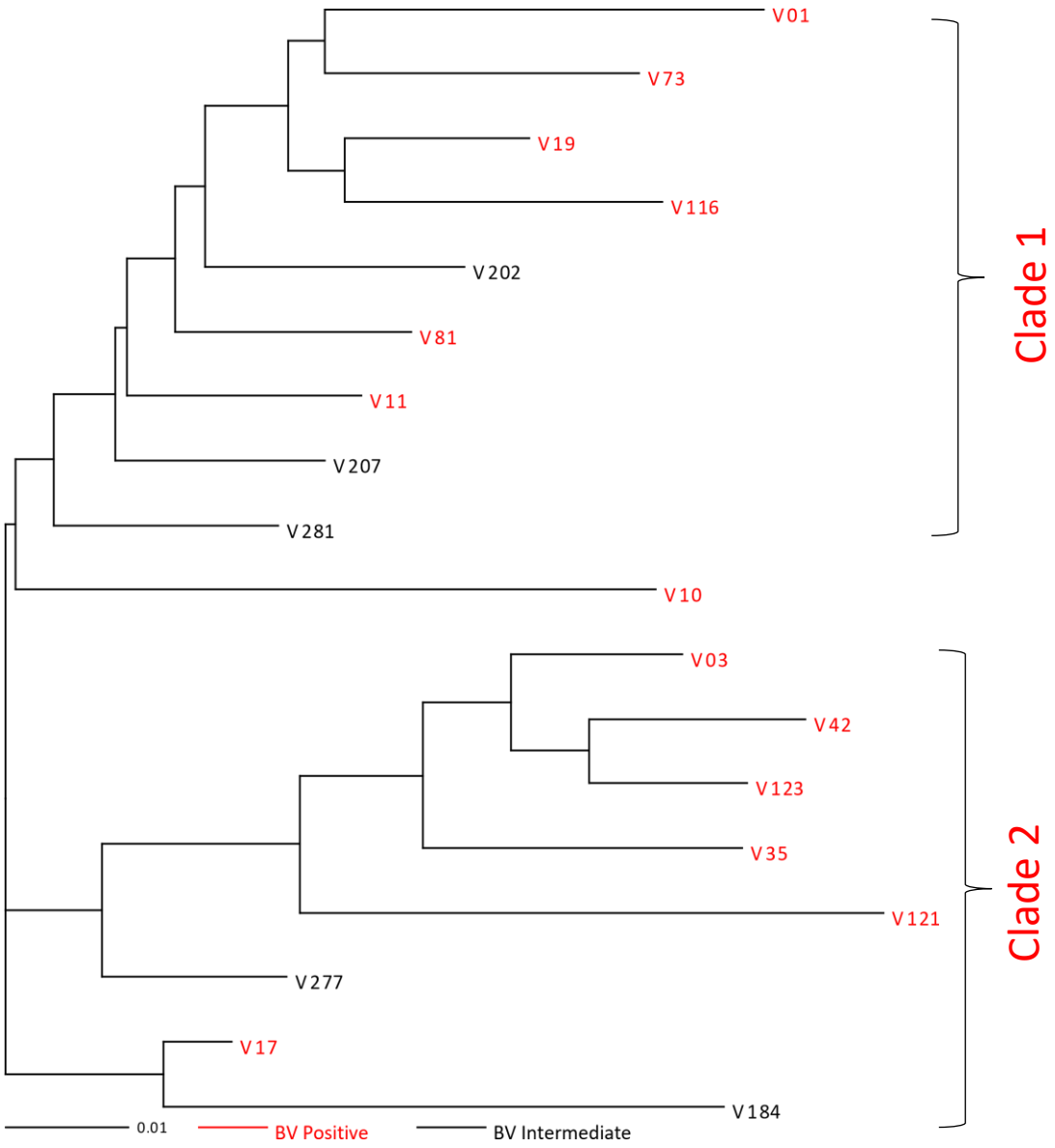


**Figure 8: Phylogenetic tree analysis of *sialidase A* of BV positive (black) and BV intermediate (red). ClustaW multiple alignment was used to create a Neighbour-joining tree with 1000 bootstrap replications. The phylogenetic tree revealed two major clades of *sialidase A* gene.**

Interestingly, the phylogenetic tree analysis (Figure 8) revealed 2 major clades of the *sialidase A* gene. Samples V01, V73, V19, V116, V202, V81, V11, V207 V281 and V10 clustered in the upper clade (Clade 1) and samples V73, V19, V184, V17, V277, V121, V35, V123, V42 and V03 clustering in the lower clade (Clade 2). The BV intermediates (in red) clustered in Clade 1 with BV positives (in black) and they all harbored a high copy number of *sialidase A*. Similar observations were made for Clade 2. Following, the phylogenetic analysis the copies of *sialidase A* obtained from (ddPCR) were compared to the genetic information obtained from sequencing (Appendix 2). Interestingly, clade 2 (V184, V17, V121, V35, V123, V42, V03) seemed to have more samples with lower copies while clade 1 contained

samples with higher copies of *sialidase A.* The average copies for clade 2 was 4,053 while the average copies for clade 1 was 22,548 (an estimated 6-fold increase). As shown in Figure 9, there is a strong correlation between the genetic sequences and the number of copies of the *sialidase A* gene.

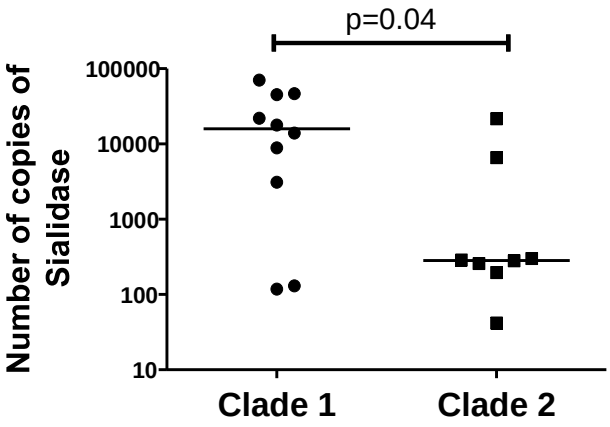


**Figure 9: Graph showing the copy numbers of *sialidase A* in relation to the genetic sequences.**

# CHAPTER FIVE

# DISCUSSION

*Gardnerella vaginalis* is strongly associated with bacterial vaginosis (BV), and is one of the most frequently isolated bacteria from women with symptoms of BV (56). Abundance of *G. vaginalis* in vaginal samples has also been associated with infertility and preterm labour (57). The role of *G. vaginalis* in vaginal disease remains incompletely understood due to its presence in both healthy and BV-type vaginal microflora. To the best of our knowledge, no studies locally have addressed nor studied the genetic diversity of intermediate BV in pregnant women thus far. To better understand the diversity of strains of *G. vaginalis*, this study investigated the genetic diversity of this pathogen from two BV states, BV positive and BV intermediate cases.

Comparative genomic analyses of 17 clinical isolates of *G. vaginalis* in all three BV states suggested that the species can be subdivided into 4 clades or that there may be multiple species of *G. vaginalis.* Ahmed et al (58) found that the degree of diversity among the strains was exceptionally high for a single species. Genomic sequencing has shown differences in virulence factors among strains of *G. vaginalis* (37). The current study investigated the diversity of the 16S rRNA of *G. vaginalis* present in BV intermediate and positive women. According to the sequence alignment of all samples analyzed, there were no genetic differences in the sequences across the BV intermediate and positive samples. In a study conducted by Eren, Zozaya *et al* (59) which explored the diversity of *G. vaginalis* 16S rRNA in the genitourinary tract of monogamous couples suggested that there may be a unique, closely related group of *G. vaginalis* oligo-types found among normal and some intermediate women. However, due the relatively limited number of normal and intermediate women, the study required these results to be confirmed by additional studies (59). Furthermore, the above-mentioned study employed the use of pyrosequencing to investigate *G. vaginalis* diversity at a low level of taxonomic discrimination. After extensive survey of the literature, we were unable to find other studies which explored the diversity of the 16S rRNA from *G. vaginalis*, thereby limiting our discussion. Therefore, the data presented in this study adds to the growing body of knowledge regarding the diversity of *G. vaginalis* especially in a pregnant population.

This study also investigated the diversity of the *sialidase A* gene, the virulence factor of *G. vaginalis*. *Sialidase A* was not detected in some samples by conventional PCR, however, ddPCR was able to detect *sialidase A* in all the samples as it allows absolute quantification of nucleic acids or absolute count of target DNA copies per sample. Locally, no studies have quantified the *sialidase A* gene of *G. vaginalis.*

A high copy number of *sialidase A* was observed for both BV positive and intermediate cases. Few studies have reported that the presence of *sialidase A* gene does not necessarily indicate the expression and/ or activity of the sialidase enzyme (60). In the current study a high copy numbers of *sialidase A* was associated with the presence of vaginal discharge. In addition, the copy numbers of *sialidase A* was significantly higher in the BV positive group when compared to the BV intermediate group. A clinical manifestation of BV is the presence of abnormal vaginal discharge, the high copy number of *Sialidase A* ,the virulence gene should be expected in the presence of discharge as revealed in this study. A future study that will be of interest is to investigate the messenger ribosomal nucleic acid (mRNA) expression levels of *Sialidase A* across the two BV states.

Upon sequencing of *sialidase A* gene of *G. vaginalis*, the phylogenetic tree analysis revealed two major clades. Interestingly, both groups, BV positive and intermediates were found across both clades. Some BV intermediate samples clustered in clade 1 with the BV positives and they all harboured a high copy number of *sialidase A*. Similar observations was made with clade 2. However, clade 2 contained samples with lower copies while clade 1 contained samples with higher copies of *sialidase A.* Clade 1 showed a strong correlation between the genetic sequences and the number of copies of the *sialidase A* gene when compared to clade 2. In a recent study in the USA where they quantified and subtyped non-cultured vaginal samples by qPCR, the presence of multiple clades had a high positive association with BV type flora (61). In our study population, although the vaginal samples were characterized by Nugent scoring criteria there was no significant association between *sialidase A* copy numbers or clades with respect to Nugent score.

Two recent studies which associated 4 clades with the Nugent scoring results had revealed that clade 4 had no association with BV, confirming the observations made by Balashov and colleagues. In addition, Janulaitiene and colleagues (62) also found that *G. vaginalis* strains of clade 1 were mostly likely to colonize BV positive women and this confirmed the findings made by Balashov et al (61, 62). Balashov and colleagues reported that clade 2 of the clades they studied was associated with intermediate vaginal flora (NS 4-6), Interestingly, Janulaitiene and collegues findings contradicted this, as they revealed that this clade was significantly more common in samples with high Nugent score (NS 7-10). These two studies used different PCR techniques, singleplex, conventional, clade specific PCR (for samples from Lithunian women) and multiplex qPCR for the samples from the USA. While in the current study we employed conventional PCR and droplet digital PCR for the quantification of *sialidase A.*

In another study of cpn60 characterization based *G. vaginalis* subgroups, subgroups of *G. vaginalis* were not evenly distributed among vaginal microbiomes diagnosed as BV positive, intermediate or normal based on Nugent score (63). Although *G. vaginalis* sequences were present in their study group, and most women harboured multiple subgroups of *G. vaginalis*, only subgroup B was significantly more abundant in BV positive compared to normal samples. Moreover analysis of pH and clue cells in the

samples they studied showed a negative correlation of pH and Nugent score and a positive correlation of clue cells and Nugent score (63).

Three studies recently reported that it is possible that *G. vaginalis* has several species with distinct roles in BV pathogenesis. Phenotypic diversity within *G. vaginalis* has also been described in terms of virulence factors, particularly production of sialidase. It was reported that there is a possible link between symptomatic BV and sialidase production and biofilm formation (32). Genetic heterogeneity within *G. vaginalis* has been demonstrated using amplified ribosomal DNA restriction analysis (ARDRA) (64). Santiago and colleagues identified three ARDRA genotypes of *G. vaginalis*, of which only two genotypes (genotypes 1 and 3) produced sialidase. A recent study analysed *G. vaginalis* clinical isolates of three subgroups (clades 1, 2, and 4) with respect to their ability to produce the toxin vaginolysin, form a biofilm, and secrete active sialidase. It was also reported that despite the presence of the *sialidase A* gene, some *G. vaginalis* isolates test negative for sialidase activity. The heterogeneity of the published studies that have been conducted on the diversity of *G. vaginalis* warrants more research in this field.

One limitation of the current study was the small number (n=13) of samples used to construct the phylogenetic tree of *sialidase A.* Even though the highly sensitive ddPCR was used to quantify the *sialidase A* copy numbers, a larger sample might produce different results of *sialidase A* gene clade distributions in association with *sialidase* copy numbers and/ or association with BV depending on their health status, race, age or geographical location. Despite this limitation, this is the first study to provide data on the diversity of *G. vaginalis* from BV positive and intermediate pregnant women. This study has provided the foundation for future studies in our research group which are now are being developed on genotyping of *G. vaginalis* from pregnant women.

# CHAPTER SIX

# CONCLUSION

The present study is the first attempt to explore the genetic diversity of strains of *G. vaginalis* in a pregnant population. *G. vaginalis* has been associated with BV, a condition that increases the risk of preterm birth thereby increasing the susceptibility to HIV acquisition. A variety of studies have failed to reveal the actual cause of BV using different techniques, especially since *G. vaginalis* has also been isolated from healthy asymptomatic women as well. This also raises questions if the genetic differences among strains might distinguish pathogenic strains from commensal strains. In the present study the 16S rRNA sequences of the *G. vaginalis* from BV intermediate and positive women showed that there is no genetic diversity in the 16S rRNA of *G. vaginalis* detected in BV positive and intermediate samples. The phylogenetic tree of *sialidase A* gene sequences of intermediate and positive BV revealed two major clades which showed differences related to *sialidase A* copy number. Some of the intermediate cases showed high copy numbers for the virulence gene and clustered with the BV positive cases. The high copy numbers of the *sialidase A* gene was associated with the presence of abnormal discharge. Lastly, the biogenetic sequences of *sialidase A* were associated with high copies numbers of the virulence factor. This calls for future studies that focus on the treatment of BV in intermediate cases. This is especially important for pregnant women since BV has been linked to considerable gynaecological and obstetric morbidity such as preterm delivery, pelvic inflammation disease and upper genital tract infections.

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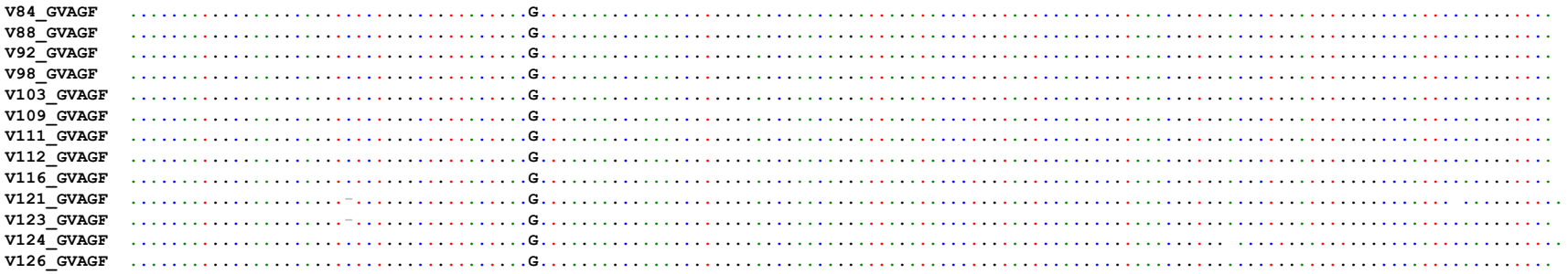
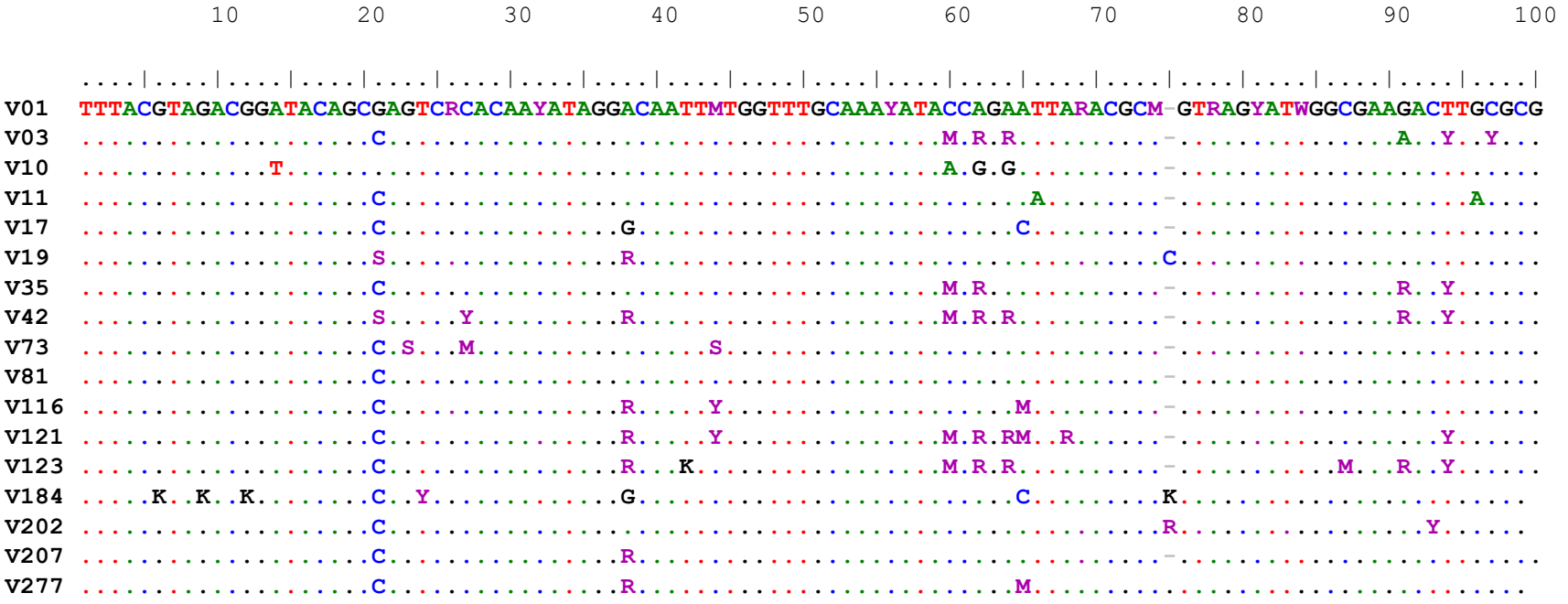
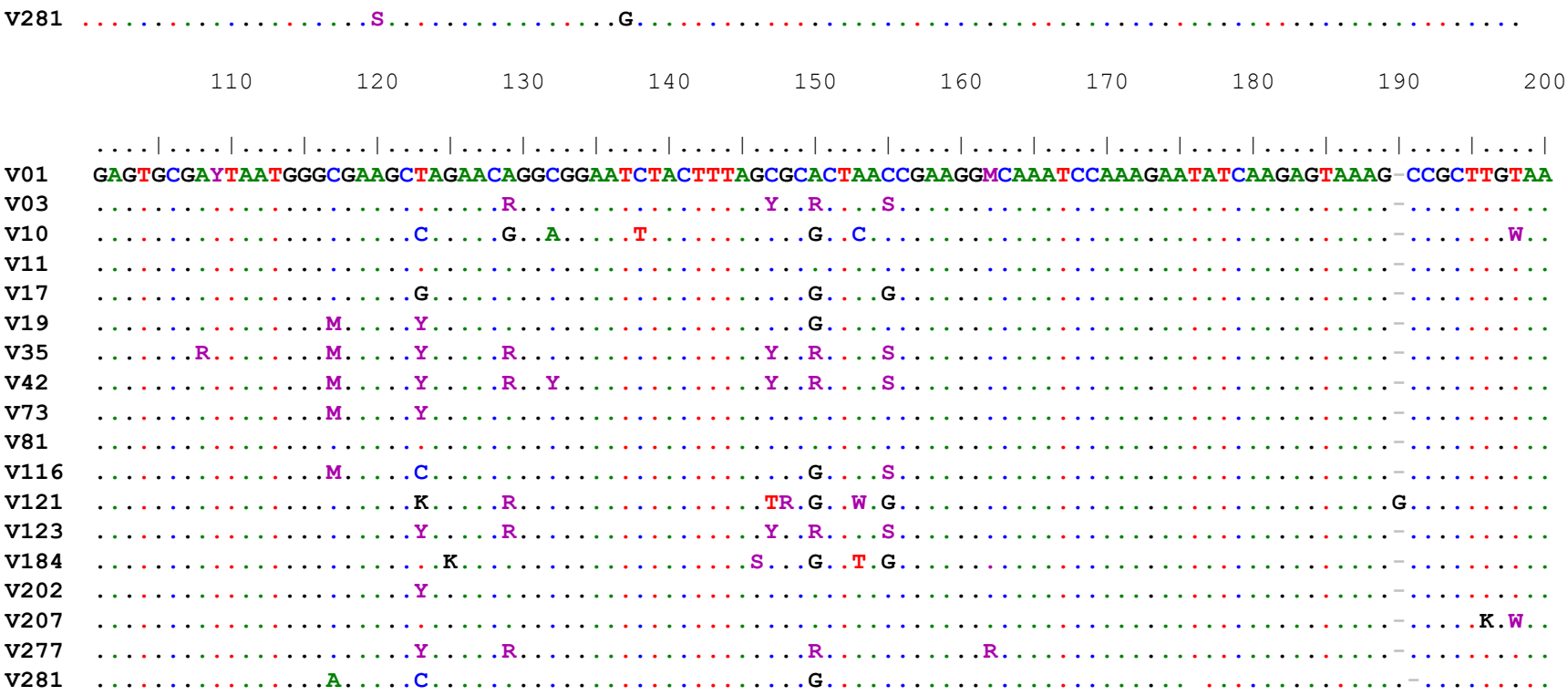
# APPENDIX

## Appendix 1: Sequence alignment of 16SrRNA *G. vaginalis.*




## Appendix 2: Sequence alignment of *sialidase A* gene




V281 .....S.....G.....
110 120 130 140 150 160 170 180 190 200
V01 GAGTGCGAYTAATGGGCGAAGCTAGAACAGGCGGAATCTACTTTAGCGCACTAACCGAAGGMCAAATCCAAAGAATATCAAGAGTAAAG-CCGCTTGTAA
V03 .....R.....Y..R....S.....-.....
V10 .....C.....G..A.....T.....G..C.....-.....W..
V11 .....-.....
V17 .....G.....G....G.....-.....
V19 .....M.....Y.....G.....-.....
V35 .....R.....M.....Y.....R.....Y..R....S.....-.....
V42 .....M.....Y.....R..Y.....Y..R....S.....-.....
V73 .....M.....Y.....-.....
V81 .....-.....
V116 .....M.....C.....G....S.....-.....
V121 .....K.....R.....TR.G..W.G.....G.....
V123 .....Y.....R.....Y..R....S.....-.....
V184 .....K.....S...G..T.G.....-.....
V202 .....Y.....-.....
V207 .....-.....K.W..
V277 .....Y.....R.....R.....R.....-.....
V281 .....A.....C.....G.....-.....