

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

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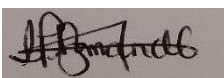
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LIST OF ABBREVIATIONS AND ACRONYMS

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) infection. 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 H₂O₂ 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 prostatitis 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).

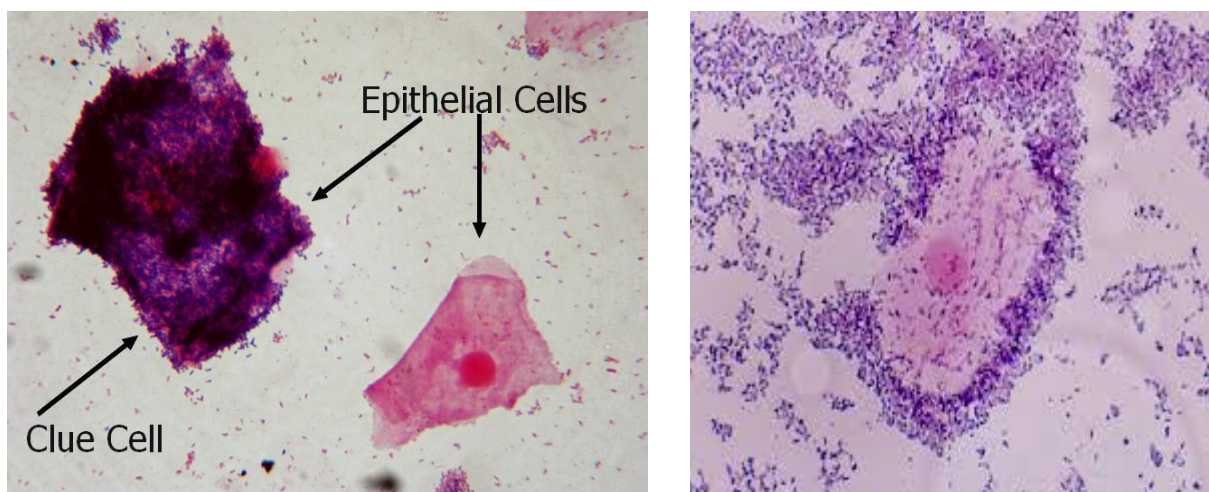


Figure 1: A: *G. vaginalis* attached to exfoliated vaginal epithelial cells (clue cells). B: *G. vaginalis* as seen under microscopy after gram staining . (Centres for Disease Control and Prevention, 2010)

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% CO₂. Optimum growth of *G. vaginalis* occurs at temperatures between 35 and 37⁰C, however, growth can occur between 25- 45⁰C . 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 neuraminidase 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 μ l 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 μ l of S2 lysis enhancer was added to the bead tube, capped and vortexed briefly. This was incubated at 95°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 μ l 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 μ l of binding buffer was added and vortexed briefly. Following this, 700 μ l 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 μ l 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°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 95°C for 10 minutes followed by 40 cycles of 95°C for 30 seconds (denaturation), annealing was performed at 55°C for 30 seconds and extension at 72°C for 30 seconds, followed by a final extension at 72°C 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°C for 10 minutes followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing was performed at 58°C for 1 minute and extension at 72°C for 2 minutes, this was followed by final extension at 72°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°C for 10 min, 40 cycles of 94°C for 30 sec and 60°C for 1 min, and 98°C 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 25-29 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).

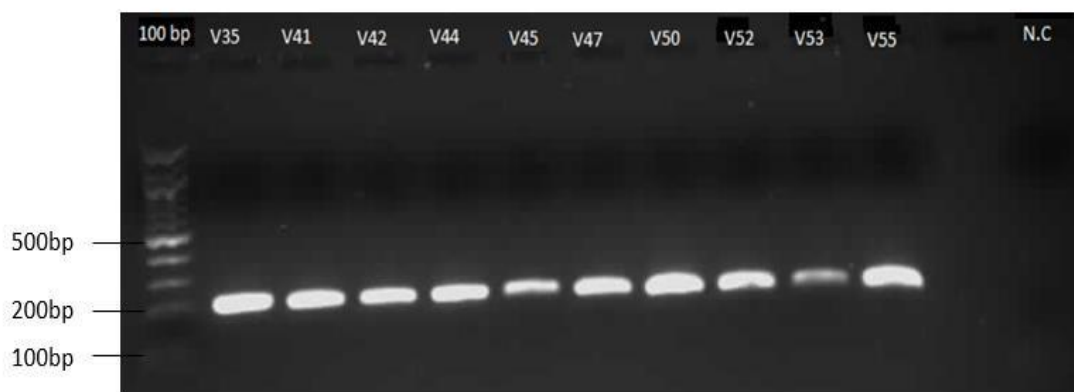


Figure 2: Agarose gel electrophoresis of selected BV positive samples (n=10). Lane 1: 100 bp molecular weight marker (Fermentas), Lane 2 – lane 10 (positive bacterial vaginosis samples), and Lane 12 Negative control (N.C).

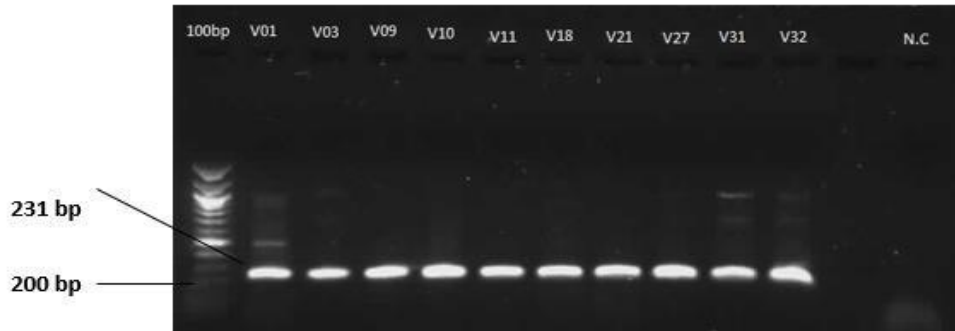


Figure 3: Agarose gel electrophoresis of selected BV positive samples (n=10) to confirm the presence of the *sialidase A* gene. Lane 1:100bp molecular weight marker (Fermentas), Lane 2-11: BV samples, and Lane 13: Negative control.



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	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	99.28	gi 1530815055 MH898666.1
V03	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	99.36	gi 1530815055 MH898666.1
V09	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	100	gi 1530815055 MH898666.1
V10	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	100	gi 1530815055 MH898666.1
V11	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	99.39	gi 1530815055 MH898666.1
V13	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	98.52	gi 1530815055 MH898666.1
V17	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	100	gi 1530815055 MH898666.1
V18	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	100	gi 1530815055 MH898666.1

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

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

V78	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	99.37	gi 1530815055 MH898666.1
V81	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	100	gi 1530815055 MH898666.1
V82	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	99.32	gi 1530815055 MH898666.1
V83	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	100	gi 1530815055 MH898666.1
V84	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	100	gi 1530815055 MH898666.1
V85	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	99.37	gi 1530815055 MH898666.1
V88	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	100	gi 1530815055 MH898666.1
V92	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	100	gi 1530815055 MH898666.1
V93	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	98.17	gi 1530815055 MH898666.1
V95	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	100	gi 1530815055 MH898666.1
V96	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	100	gi 1530815055 MH898666.1
V98	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	99.39	gi 1530815055 MH898666.1
V101	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	99.36	gi 1530815055 MH898666.1
V102	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	98.79	gi 1530815055 MH898666.1
V103	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	100	gi 1530815055 MH898666.1
V109	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	100	gi 1530815055 MH898666.1

V111	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	100	gi1530815055 MH898666.1
V112	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	100	gi1530815055 MH898666.1

V116	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	100	gi1530815055 MH898666.1
V117	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	98.78	gi1530815055 MH898666.1
V121	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	99.36	gi1530815055 MH898666.1
V123	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	99.36	gi1530815055 MH898666.1
V124	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	100	gi1530815055 MH898666.1
V126	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	99.39	gi1530815055 MH898666.1
V184	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	99.36	gi1530815055 MH898666.1
V202	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	100	gi1530815055 MH898666.1
V206	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	98.58	gi1530815055 MH898666.1
V207	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	99.36	gi1530815055 MH898666.1
V239	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	99.39	gi1530815055 MH898666.1
V240	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	98.78	gi1530815055 MH898666.1
V250	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	99.36	gi1530815055 MH898666.1
V258	<i>G. vaginalis</i> strain UGent25.49 16S rRNA, partial sequence	98.78	gi1530815055 MH898666.1

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

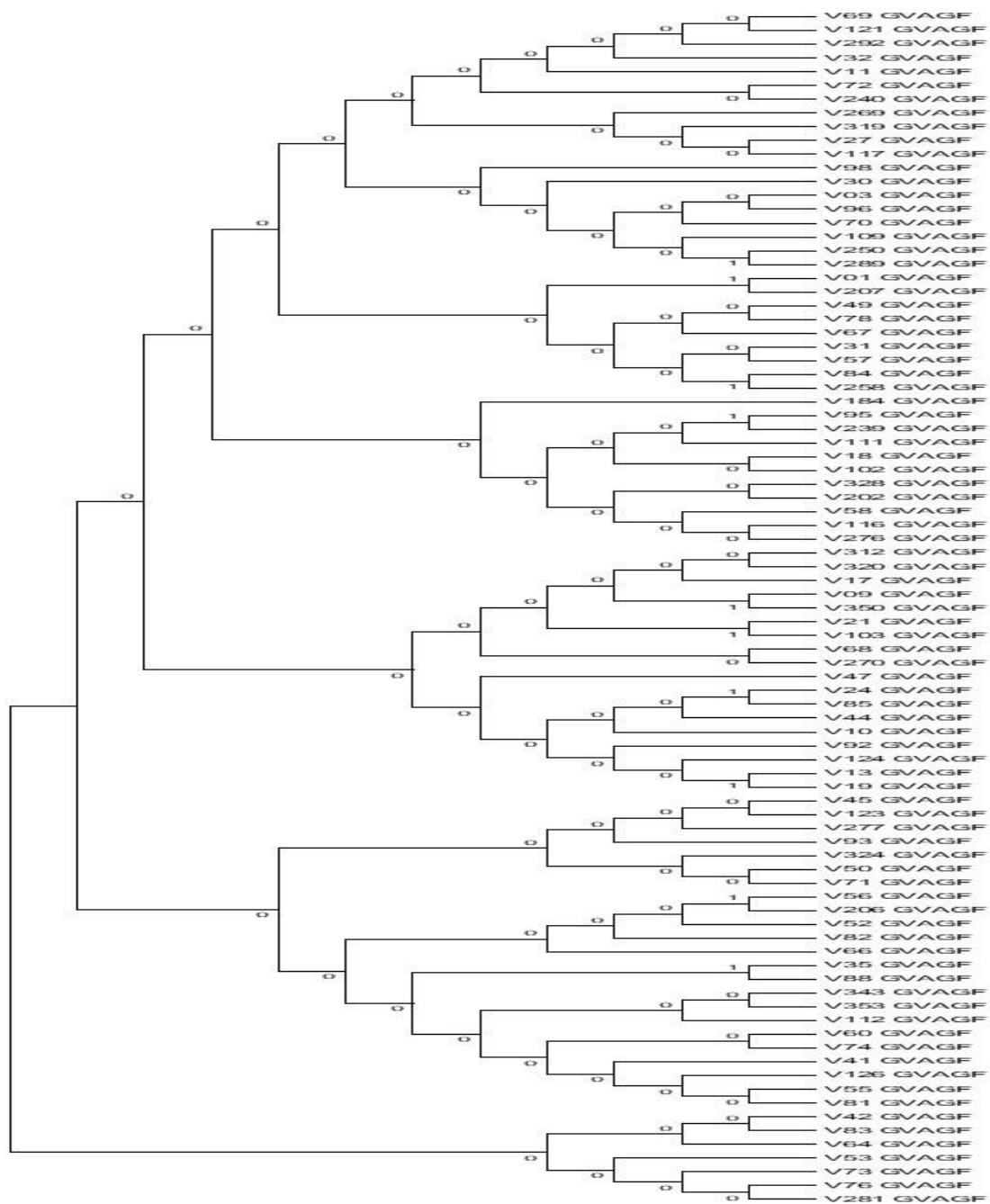


Figure 5: The evolutionary history was inferred using the UPGMA method. The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in 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		Positive Droplets	Negative droplets	Accepted Droplets	Threshold	BV status
			Confidence	Confidence					
			Max	Min					
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	1,2	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.

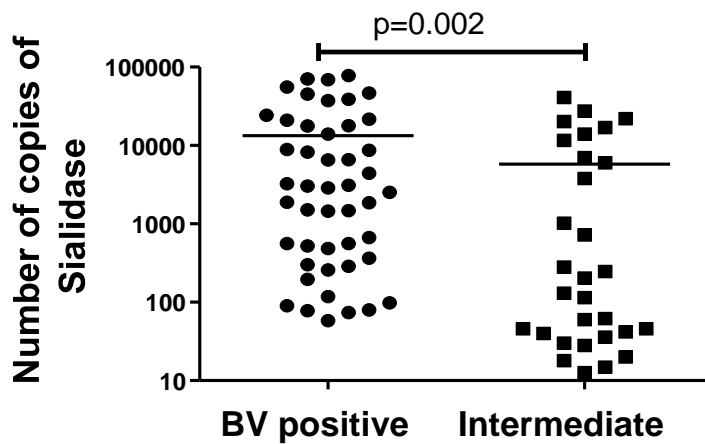


Figure 6: *Sialidase A* copy numbers associated with the BV positive and BV intermediate groups, $p=0.002$.

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.

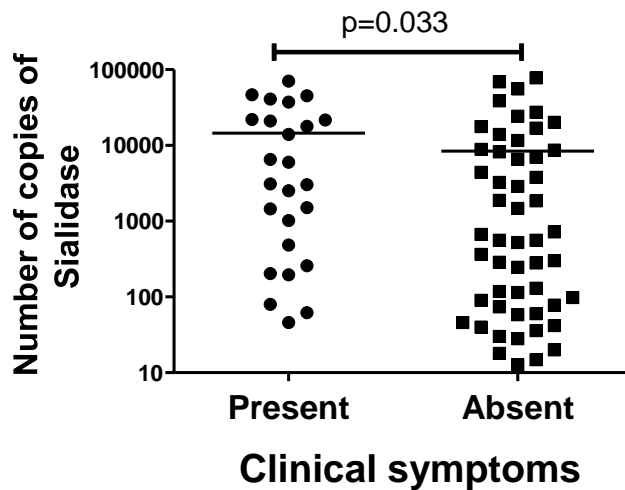


Figure 7: Association of *sialidase A* copy numbers with the presence or absence of BV clinical symptoms (abnormal vaginal discharge); $p= 0.033$.

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 V281 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).

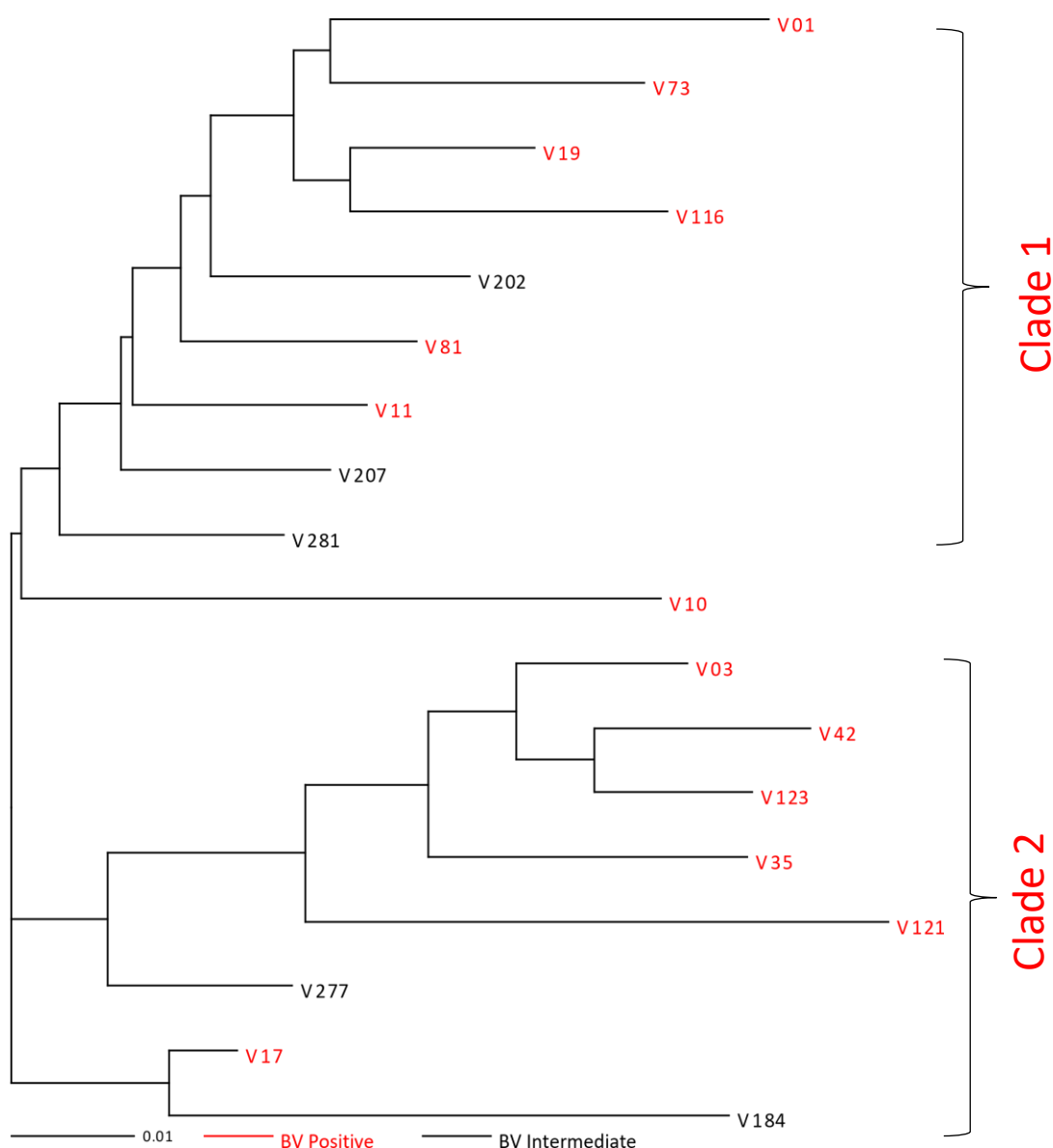


Figure 8: Phylogenetic tree analysis of *sialidase A* of BV positive (black) and BV intermediate (red). ClustaW multiple alignment was used to create a Neighbour-joining tree with 1000 bootstrap replications. The phylogenetic tree revealed two major clades of *sialidase A* gene.

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.

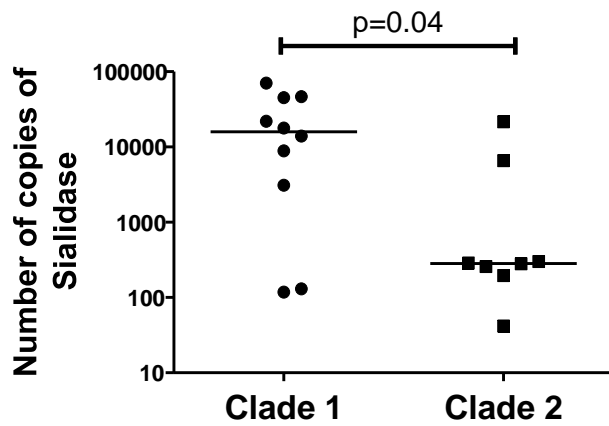


Figure 9: Graph showing the copy numbers of *sialidase A* in relation to the genetic sequences.

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 to 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 colleagues 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 Lithuanian 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.

BIBLIOGRAPHY

1. Oduyebo OO, Anorlu RI, Ogunsola FT. The effects of antimicrobial therapy on bacterial vaginosis in non-pregnant women. *Cochrane Database of Systematic Reviews*. 2009(3).
2. Forsum U, Holst E, Larsson P-G, Vasquez A, Jakobsson T, Mattsby-Baltzer I. Bacterial vaginosis—a microbiological and immunological enigma. *Apmis*. 2005;113(2):81-90.
3. Turovskiy Y, Sutyak Noll K, Chikindas ML. The aetiology of bacterial vaginosis. *Journal of applied microbiology*. 2011;110(5):1105-28.
4. Swidsinski A, Mendling W, Loening-Baucke V, Swidsinski S, Dörffel Y, Scholze J, et al. An adherent *Gardnerella vaginalis* biofilm persists on the vaginal epithelium after standard therapy with oral metronidazole. *American journal of obstetrics and gynecology*. 2008;198(1):97. e1-. e6.
5. Josey W, Schwebke J. The polymicrobial hypothesis of bacterial vaginosis causation: a reassessment. *International journal of STD & AIDS*. 2008;19(3):152-4.
6. Harwich MD, Alves JM, Buck GA, Strauss JF, Patterson JL, Oki AT, et al. Drawing the line between commensal and pathogenic *Gardnerella vaginalis* through genome analysis and virulence studies. *BMC genomics*. 2010;11(1):375.
7. Swidsinski A, Mendling W, Loening-Baucke V, Ladhoff A, Swidsinski S, Hale LP, et al. Adherent biofilms in bacterial vaginosis. *Obstetrics & Gynecology*. 2005;106(5):1013-23.
8. Machado A, Cerca N. Influence of biofilm formation by *Gardnerella vaginalis* and other anaerobes on bacterial vaginosis. *The Journal of infectious diseases*. 2015;212(12):1856-61.
9. Kenyon CR, Osbak K. Recent progress in understanding the epidemiology of bacterial vaginosis. *Current Opinion in Obstetrics and Gynecology*. 2014;26(6):448-54.
10. Antonio MA, Hawes SE, Hillier SL. The identification of vaginal *Lactobacillus* species and the demographic and microbiologic characteristics of women colonized by these species. *Journal of Infectious Diseases*. 1999;180(6):1950-6.
11. Suau A, Bonnet R, Sutren M, Godon J-J, Gibson GR, Collins MD, et al. Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. *Appl Environ Microbiol*. 1999;65(11):4799-807.
12. Pleckaityte M, Janulaitiene M, Lasickiene R, Zvirbliene A. Genetic and biochemical diversity of *Gardnerella vaginalis* strains isolated from women with bacterial vaginosis. *FEMS Immunology & Medical Microbiology*. 2012;65(1):69-77.

13. Leitich H, Kiss H. Asymptomatic bacterial vaginosis and intermediate flora as risk factors for adverse pregnancy outcome. *Best practice & research Clinical obstetrics & gynaecology*. 2007;21(3):375-90.
14. Romero R, Chaiworapongsa T, Kuivaniemi H, Tromp G. Bacterial vaginosis, the inflammatory response and the risk of preterm birth: a role for genetic epidemiology in the prevention of preterm birth. *American Journal of Obstetrics & Gynecology*. 2004;190(6):1509-19.
15. TAYLOR E, Phillips I. The identification of *Gardnerella vaginalis*. *Journal of medical microbiology*. 1983;16(1):83-92.
16. Catlin BW. *Gardnerella vaginalis*: characteristics, clinical considerations, and controversies. *Clinical Microbiology Reviews*. 1992;5(3):213-37.
17. PIOT P, VAN DYCK E, GOODFELLOW M, FALKOW S. A taxonomic study of *Gardnerella vaginalis* (*Haemophilus vaginalis*) Gardner and Dukes 1955. *Microbiology*. 1980;119(2):373-96.
18. Edmunds P. *Haemophilus vaginalis*: morphology, cultural characters and viability. *The Journal of pathology and bacteriology*. 1960;79(2):273-84.
19. Dunkelberg JR WE, Skaggs R, Kellogg JR DS. A study and new description of *Corynebacterium vaginale* (*Haemophilus vaginalis*). *American journal of clinical pathology*. 1970;53(3):370-7.
20. Greenwood J, Pickett M. 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 Microbiology*. 1980;30(1):170-8.
21. Taylor-Robinson D. The bacteriology of *Gardnerella vaginalis*. *Scandinavian journal of urology and nephrology Supplementum*. 1984;86:41-55.
22. Jolly J. Minimal criteria for the identification of *Gardnerella vaginalis* isolated from the vagina. *Journal of clinical pathology*. 1983;36(4):476-8.
23. Harper J, Davis G. Cell wall analysis of *Gardnerella vaginalis* (*Haemophilus vaginalis*). *International Journal of Systematic Bacteriology*. 1982;32(1):48-50.
24. Scott TG, Curran B, Smyth CJ. Electron microscopy of adhesive interactions between *Gardnerella vaginalis* and vaginal epithelial cells, McCoy cells and human red blood cells. *Microbiology*. 1989;135(3):475-80.

25. Davies P, Cornwell MM, Johnson J, Reggianni A, Myers M, Murtaugh M. Studies on the effects of dansylcadaverine and related compounds on receptor-mediated endocytosis in cultured cells. *Diabetes Care*. 1984;7(Suppl 1):35-41.
26. Moss CW, Dunkelberg W. Volatile and cellular fatty acids of *Haemophilus vaginalis*. *Journal of bacteriology*. 1969;100(1):544-6.
27. Griffiths E. Iron and bacterial virulence—a brief overview. *Biology of metals*. 1991;4(1):7-13.
28. Hall-Stoodley L, Stoodley P, Kathju S, Høiby N, Moser C, William Costerton J, et al. Towards diagnostic guidelines for biofilm-associated infections. *FEMS Immunology & Medical Microbiology*. 2012;65(2):127-45.
29. Schwebke JR, Flynn MS, Rivers CA. Prevalence of *Gardnerella vaginalis* among women with lactobacillus-predominant vaginal flora. *Sex Transm Infect*. 2014;90(1):61-3.
30. Muzny CA, Schwebke JR. Pathogenesis of bacterial vaginosis: discussion of current hypotheses. *The Journal of infectious diseases*. 2016;214(suppl_1):S1-S5.
31. Schwebke JR, Muzny CA, Josey WE. Role of *Gardnerella vaginalis* in the pathogenesis of bacterial vaginosis: a conceptual model. *The Journal of infectious diseases*. 2014;210(3):338-43.
32. dos Santos Santiago GL, Deschaght P, El Aila N, Kiama TN, Verstraelen H, Jefferson KK, et al. *Gardnerella vaginalis* comprises three distinct genotypes of which only two produce sialidase. *American journal of obstetrics and gynecology*. 2011;204(5):450. e1-. e7.
33. Von Nicolai H, Hammann R, Salehnia S, Zilliken F. A newly discovered sialidase from *Gardnerella vaginalis*. *Zentralblatt für Bakteriologie, Mikrobiologie und Hygiene Series A: Medical Microbiology, Infectious Diseases, Virology, Parasitology*. 1984;258(1):20-6.
34. Briselden AM, Moncla BJ, Stevens CE, Hillier SL. Sialidases (neuraminidases) in bacterial vaginosis and bacterial vaginosis-associated microflora. *Journal of clinical microbiology*. 1992;30(3):663-6.
35. Gelber SE, Aguilar JL, Lewis KL, Ratner AJ. Functional and phylogenetic characterization of Vaginolysin, the human-specific cytolysin from *Gardnerella vaginalis*. *Journal of bacteriology*. 2008;190(11):3896-903.
36. Teixeira G, Soares-Brandao K, Branco K, Sampaio J, Nardi R, Mendonça M, et al. Antagonism and synergism in *Gardnerella vaginalis* strains isolated from women with bacterial vaginosis. *Journal of medical microbiology*. 2010;59(8):891-7.

37. Yeoman CJ, Yildirim S, Thomas SM, Durkin AS, Torralba M, Sutton G, et al. Comparative genomics of *Gardnerella vaginalis* strains reveals substantial differences in metabolic and virulence potential. *PloS one*. 2010;5(8):e12411.
38. Mehta SD, Donovan B, Weber KM, Cohen M, Ravel J, Gajer P, et al. The vaginal microbiota over an 8-to 10-year period in a cohort of HIV-infected and HIV-uninfected women. *PloS one*. 2015;10(2):e0116894.
39. Schellenberg J, Links MG, Hill JE, Dumonceaux TJ, Peters GA, Tyler S, et al. Pyrosequencing of the chaperonin-60 universal target as a tool for determining microbial community composition. *Appl Environ Microbiol*. 2009;75(9):2889-98.
40. Hummelen R, Fernandes AD, Macklaim JM, Dickson RJ, Chagalucha J, Gloor GB, et al. Deep sequencing of the vaginal microbiota of women with HIV. *PloS one*. 2010;5(8):e12078.
41. Abbai NS, Nyirenda M, Naidoo S, Ramjee G. Prevalent herpes simplex virus-2 increases the risk of incident bacterial vaginosis in women from South Africa. *AIDS and behavior*. 2018:1-9.
42. Abbai NS, Wand H, Ramjee G. Socio-demographic and behavioural characteristics associated with HSV-2 sero-prevalence in high risk women in KwaZulu-Natal. *BMC research notes*. 2015;8(1):185.
43. Onderdonk AB, Delaney ML, Fichorova RN. The human microbiome during bacterial vaginosis. *Clinical microbiology reviews*. 2016;29(2):223-38.
44. Leitich H, Bodner-Adler B, Brunbauer M, Kaider A, Egarter C, Husslein P. Bacterial vaginosis as a risk factor for preterm delivery: a meta-analysis. *American journal of obstetrics and gynecology*. 2003;189(1):139-47.
45. Bretelle F, Rozenberg P, Pascal A, Favre R, Bohec C, Loundou A, et al. High *Atopobium vaginae* and *Gardnerella vaginalis* vaginal loads are associated with preterm birth. *Clinical Infectious Diseases*. 2014;60(6):860-7.
46. MacIntyre DA, Chandiramani M, Lee YS, Kindinger L, Smith A, Angelopoulos N, et al. The vaginal microbiome during pregnancy and the postpartum period in a European population. *Scientific reports*. 2015;5:8988.
47. Donders G, Van Calsteren K, Bellen G, Reybrouck R, Van den Bosch T, Riphagen I, et al. Predictive value for preterm birth of abnormal vaginal flora, bacterial vaginosis and aerobic vaginitis during the first trimester of pregnancy. *BJOG: An International Journal of Obstetrics & Gynaecology*. 2009;116(10):1315-24.

48. Bradshaw C, Morton A, Garland S, Horvath L, Kuzevska I, Fairley C. Evaluation of a point-of-care test, BVBlue, and clinical and laboratory criteria for diagnosis of bacterial vaginosis. *Journal of clinical microbiology*. 2005;43(3):1304-8.
49. Spiegel CA, Amsel R, Holmes K. Diagnosis of bacterial vaginosis by direct gram stain of vaginal fluid. *Journal of clinical microbiology*. 1983;18(1):170-7.
50. Nugent RP, Krohn MA, Hillier SL. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. *Journal of clinical microbiology*. 1991;29(2):297-301.
51. Ferris MJ, Maszta A, Aldridge KE, Fortenberry JD, Fidel PL, Martin DH. Association of *Atopobium vaginae*, a recently described metronidazole resistant anaerobe, with bacterial vaginosis. *BMC infectious diseases*. 2004;4(1):5.
52. Fredricks DN, Fiedler TL, Thomas KK, Oakley BB, Marrazzo JM. Targeted PCR for detection of vaginal bacteria associated with bacterial vaginosis. *Journal of clinical microbiology*. 2007;45(10):3270-6.
53. Menard J-P, Fenollar F, Henry M, Bretelle F, Raoult D. Molecular quantification of *Gardnerella vaginalis* and *Atopobium vaginae* loads to predict bacterial vaginosis. *Clinical Infectious Diseases*. 2008;47(1):33-43.
54. Gaydos CA, Beqaj S, Schwebke JR, Lebed J, Smith B, Davis TE, et al. Clinical validation of a test for the diagnosis of vaginitis. *Obstetrics and gynecology*. 2017;130(1):181.
55. Landers DV, Wiesenfeld HC, Heine RP, Krohn MA, Hillier SL. Predictive value of the clinical diagnosis of lower genital tract infection in women. *American journal of obstetrics and gynecology*. 2004;190(4):1004-8.
56. Schellenberg JJ, Links MG, Hill JE, Dumonceaux TJ, Kimani J, Jaoko W, et al. Molecular definition of vaginal microbiota in East African commercial sex workers. *Appl Environ Microbiol*. 2011;77(12):4066-74.
57. Menard JP, Mazouni C, Salem-Cherif I, Fenollar F, Raoult D, Boubli L, et al. High vaginal concentrations of *Atopobium vaginae* and *Gardnerella vaginalis* in women undergoing preterm labor. *Obstetrics & Gynecology*. 2010;115(1):134-40.
58. Ahmed A, Earl J, Retchless A, Hillier SL, Rabe LK, Cherpes TL, et al. Comparative genomic analyses of seventeen clinical isolates of *Gardnerella vaginalis* provides evidence of multiple genetically isolated clades consistent with sub-speciation into genovars. *Journal of bacteriology*. 2012;JB. 00056-12.

59. Eren AM, Zozaya M, Taylor CM, Dowd SE, Martin DH, Ferris MJ. Exploring the diversity of *Gardnerella vaginalis* in the genitourinary tract microbiota of monogamous couples through subtle nucleotide variation. *PloS one*. 2011;6(10):e26732.
60. Schellenberg JJ, Jayaprakash TP, Gamage NW, Patterson MH, Vaneechoutte M, Hill JE. *Gardnerella vaginalis* subgroups defined by cpn60 sequencing and sialidase activity in isolates from Canada, Belgium and Kenya. *PloS one*. 2016;11(1):e0146510.
61. Balashov SV, Mordechai E, Adelson ME, Gyax SE. Identification, quantification and subtyping of *Gardnerella vaginalis* in noncultured clinical vaginal samples by quantitative PCR. *Journal of medical microbiology*. 2014;63(2):162-75.
62. Janulaitiene M, Paliulyte V, Grinceviciene S, Zakareviciene J, Vladisauskiene A, Marcinkute A, et al. Prevalence and distribution of *Gardnerella vaginalis* subgroups in women with and without bacterial vaginosis. *BMC infectious diseases*. 2017;17(1):394.
63. Jayaprakash TP, Schellenberg JJ, Hill JE. Resolution and characterization of distinct cpn60-based subgroups of *Gardnerella vaginalis* in the vaginal microbiota. *PLoS One*. 2012;7(8):e43009.
64. Ingiani A, Petruzzelli S, Morandotti G, Pompei R. Genotypic differentiation of *Gardnerella vaginalis* by amplified ribosomal DNA restriction analysis (ARDRA). *FEMS Immunology & Medical Microbiology*. 1997;18(1):61-6.

APPENDIX

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




```

V84_GVAGF .....G.....
V88_GVAGF .....G.....
V92_GVAGF .....G.....
V98_GVAGF .....G.....
V103_GVAGF .....G.....
V109_GVAGF .....G.....
V111_GVAGF .....G.....
V112_GVAGF .....G.....
V116_GVAGF .....G.....
V121_GVAGF .....G.....
V123_GVAGF .....G.....
V124_GVAGF .....G.....
V126_GVAGF .....G.....

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Appendix 2: Sequence alignment of *sialidase A* gene

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          10      20      30      40      50      60      70      80      90     100
V01  TTTACGTAGACGGATACAGCGAGTCRCACAAYATAGGACAATTMTGGTTTGCAAAYATACCAGAATTARACGCM-GTRAGYATWGGCGAAGACTTGCGCG
V03  .....C.....M.R.R.....A.Y.Y...
V10  .....T.....A.G.G.....
V11  .....C.....A.....A...
V17  .....C.....G.....C.....
V19  .....S.....R.....C.....
V35  .....C.....M.R.....R.Y...
V42  .....S.Y.R.S.....M.R.R.....R.Y...
V73  .....C.S.M.....S.....
V81  .....C.....
V116 .....C.....R.Y.....M.....
V121 .....C.....R.Y.....M.R.R.M.R.....Y...
V123 .....C.....R.K.....M.R.R.....M.R.Y...
V184 .....K.K.K.....C.Y.....G.....C.....K.....
V202 .....C.....R.....R.....Y...
V207 .....C.....R.....
V277 .....C.....R.....M.....

```

V281S.....G.....

110 120 130 140 150 160 170 180 190 200

.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|

V01 **GAGTGC****GA****YTAAT****TGGGCGAAG****CTAGA****ACAGGCCGAATCTAC****TTTAGCG****CAC****TAACCGAAG****MC****AAATCCA****AGAATATCAAGAGTAAAG****CCGCTTGTA****A**

V03R.....Y.R..S.

V10C..G.A..T.....G.C.....W.

V11|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|

V17G.....G..G.

V19M..Y.....G.

V35R..M..Y..R.....Y.R..S.

V42M..Y..R.Y.....Y.R..S.

V73M..Y.....

V81|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|

V116M..C.....G..S.

V121K..R.....TR.G..W.G.....G.

V123Y..R.....Y.R..S.

V184K.....S..G..T.G.....

V202Y.....

V207|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|

V277Y..R.....R.....R.

V281A..C.....G.....