METAGENOMIC CHARACTERISATION OF VAGINAL FLORA AND THE RELATIONSHIP OF BACTERIOPHAGE, *BIFIDOBACTERIUM* AND *OENOCOCCUS* WITH BACTERIAL VAGINOSIS IN HIV INFECTED WOMEN

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Metagenomic Characterisation of Vaginal Flora and the Relationship of Bacteriophage, *Bifidobacterium* and *Oenococcus* with Bacterial Vaginosis in HIV Infected Women

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

Signature …….. Date……...

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This thesis has been submitted for examination with our approval as University Supervisors.

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KEMRI, Kenya

DEDICATION

This work is dedicated to John Michuki Ngondi, Mrs Jane W. Michuki, Leonard Muriuki Ngondi, Edith K. Muriuki and Pamela Muringo. Thank you all.

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AIDS	Aquired Immunodeficiency Disease Syndrome
BV	Bacterial vaginosis
BVAB	Bacterial Vaginosis Associated Bacterium
CDC	Centres for Disease Control and Prevention
CIN	Cervical Intraepithelial Neoplasia
CMR	Center for Microbiology Research
CVL	Cervicovaginal Lavage
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic acid
ERC	Ethical Review Committee
GBS	Group B Streptococci
H_2O_2	Hydrogen peroxide
HIV-1	Human Immunodeficiency Virus Type 1
IVF	*In Vitro* Fertilization
KEMRI	Kenya Medical Research Institute
KOH	Potasium Hydroxide
OIF	Oil Immersion field
OMA	Osmetech Microbial Analyser
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFGE	Pulse Fluid Gel Electrophoresis
PID	Pelvic Inflammatory Diseases
RAPD	Randomly Amplified Polymorphic DNA

rRNA	ribosomal Ribonucleic acid
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SSC	Scientific Steering Committee
STD	Sexually Transmitted Disease
STI	Sexually Transmitted Infections
UCSF	University of California, San Francisco
USA	United States of America
WHO	World Health Organization

ABSTRACT

Bacterial vaginosis (BV) is a highly prevalent condition and the most common cause of abnormal vaginal discharge. Despite the high prevalence of BV and associations with other infections such as HIV-1 and obstetric and gynecological morbidity associated with it, the etiology of BV is still not clearly understood. It has been hypothesized that some unknown influences cause a significant decline in hydrogen peroxide producing lactobacilli allowing the overgrowth of anaerobic bacteria. The organisms implicated with BV have mainly been isolated by culture methods. The present study was done in two phases where the first was a pilot study done in California with the aim of determining the novel and predominant culturable and unculturable microorganisms associated with BV. To achieve this, micro-array and shot-gun sequencing techniques were used. Simpler techniques were also developed in phase one part of the study. The second phase was done in Kenya with the aim to determine the prevalence of BV among women infected with HIV and determine the association of the condition with bacteriophages and the specific microorganisms identified in phase one study. This was achieved using multiplex and simplex PCR with organism's specific primers. The pilot study in California identified two main organisms that had a relationship with BV presence or absence. *Oenococcus oeni* were present only in BV negative samples while *Bifidobacterium* species were significantly associated with BV positive samples ($p=0.0472$, Fisher's exact test). Bacteriophage type A2 was detected in 2 of 14 (14%) BV negative samples. Unculturable bacteria were over 90% of the total bacteria identified by sequencing. In Kenya, BV prevalence showed a declining trend while CD4 cells count increased with visit count. *Oenococcus oeni* was not detected in any sample. Bifidobacteria

were present in 39/250 (16%) of BV positive and in 19/250 (8%) BV negative samples. Though higher in BV positive the difference was not statistically significant ($p>0.05$; Chi-square). Bacteriophages Bradley types A1, A2, B1 and B2 were detected in BV positive and negative samples. Bacteriophage Bradley type B2 was detected at significantly higher rates in BV positive samples than in BV negative samples ($p<0.05$; Fisher's exact test). The use of different methods; shot-gun sequencing versus specific PCR may not have contributed to the differences of *Oenococcus* detection since, both methods are known to have high sensitivities with detection limits of 1 pg μl^{-1} chromosomal DNA having been reported. Identification of *Oenococcus* and unculturable bacteria confirms that shot gun sequencing is an appropriate technique for identification of novel and unculturable organisms associated with BV. In addition, detection of BV associated organisms by PCR provides an effective screening method for BV. *Bifidobacterium* species are variably associated with presence or absence of BV and phylogenetic analysis shows some are close to BV related organisms while some are not. This indicates the likelihood that there exists variant species of Bifidobacteria. This study therefore concludes that novel and unculturable bacteria are the largest population of microorganisms in the vagina of women with and without BV.

CHAPTER ONE

1.0 Introduction

Bacterial vaginosis (BV) is a polymicrobial syndrome that appears to represent an interruption of the vaginal ecosystem that is normally predominated by *Lactobacillus* species (Eschenbach, 1993; Hillier and Homes, 1999; Ferris *et al.*, 2004). BV is a highly prevalent condition and the most common cause of abnormal vaginal discharge (Sobel, 2005). Women with BV may have a malodorous vaginal discharge or local irritation, but about half of the women with diagnosable BV have no clear symptoms (Amsel *et al*., 1983). Some women do not report abnormal vaginal discharge, but the abnormal discharge is usually noted on examination by a clinician. This is an indication that many women with BV are not aware or considers their discharge to be within normal bounds (Srinivasan and Fredricks, 2008). The high prevalence of BV and the lack of symptoms in a substantial fraction of affected women lead to the question whether BV should be considered a normal variant of the vaginal microbiota or a disease (Reid and Bocking, 2003). For women affected by severe symptomatic BV as manifested by profuse vaginal discharge and less frequently by local burning or itching, there is little question that they have a disease (Srinivasan and Fredricks, 2008).

Despite the high prevalence of BV and implications and associations with other infections such as HIV-1 and obstetric and gynecological morbidity associated with it, the etiology of BV is still not clearly understood (Hooton *et al*., 1991; Bukusi *et al.*, 2006). BV is associated with reduction in *Lactobacillus* spp. and large increases in anaerobic gram-negative rods, *Gardnerella* species and genital mycoplasmas

(Hooton *et al.*, 1991; Eschenbach, 1993). Risk factors for the development of BV include douching, use of spermicides, antibiotics, HIV-1-infection, poor male genital hygiene and lack of male circumcision (Hutchinson *et al.*, 2007; Heng *et al.*, 2010).

It has been hypothesized that some unknown influences cause a significant decline in hydrogen peroxide producing lactobacilli thus, allowing the overgrowth of anaerobic bacteria (Vallor *et al*., 2001). The production of hydrogen peroxide may enhance persistence of *Lactobacillus* through direct inhibition of other bacteria of the vaginal flora; alternatively, hydrogen peroxide producing strains of lactobacilli may have surface molecules important in adherence that are co-regulated with the genes for hydrogen peroxide production (Vallor *et al*., 2001).

Temperate bacteriophages have been isolated from vaginal lactobacilli and associated with the cascade leading to BV (Kiliç *et al*., 2001). This is a significant observation since bacteriophages are known to alter host bacterial properties that are relevant to all stages of the infectious processes such as bacterial adhesion, colonization, invasion, exotoxin production, sensitivity to antibiotics and invasion into human tissues (Wagner and Waldor, 2002). This may suggest that the temperate phage isolated in Kilic *et al*. (2001) study may also have beneficial effects to the vaginal *Lactobacillus* such as production of toxic substances against other bacteria.

The present study therefore aims at determining the prevalent organisms associated with BV and also to determine the prevalence of BV among women infected with

HIV and determine the association of the condition with bacteriophages, bifidobacteria and *Oenococcus*.

1.1 Literature Review

1.1.1 Bacterial Vaginosis

Bacterial vaginosis which was earlier referred to as nonspecific vaginitis was recognized by Gardner and Duke as a vaginal syndrome over 50 years ago (Gardner and Dukes, 1955). Despite the early recognition of BV it continues to be the most prevalent vaginal disorder in adult women worldwide (Cauci *et al.*, 2002; Rossi *et al.*, 2009). The etiology and clinical courses of BV are not well defined or understood (Dumonceaux *et al*., 2009). Several findings suggest that BV is a synergistic polymicrobial condition which is characterized by a decrease in lactobacilli and an increase in several facultative and/or strictly anaerobic micro-organisms in the vagina (Eschenbach, 1993; Thorsen *et al*., 1998; Verhelst *et al*., 2004). Strong associations have been made between the syndrome and the presence of particular bacterial species, such as *Gardnerella vaginalis* and *Prevotella* sp. (Hillier and Homes, 1999). The normal vaginal flora consists predominantly of lactobacilli (Eschenbach, 1993b). Lactobacilli are Gram-positive lactic acid bacteria (LAB) with a low G+C content, are acid-tolerant and are nonspore-forming rods. They are also aero-tolerant or anaerobic and are nutritionally fastidious (Claesson *et al.*, 2007). Their natural habitat varies widely, from food, plants and sewage, to the oral, genital and gastrointestinal (GI) tracts of humans and animals (Claesson *et al.*, 2007). In the vagina, lactobacilli are a natural host defense due to the production of

lactic acid and hydrogen peroxide. Lactic acid maintains the vaginal pH below 4.5, and hydrogen peroxide inhibits the growth of non catalase producing microorganisms (Hillier and Homes, 1999).

Women with the condition BV loose many *Lactobacillus* species except *Lactobacillus iners* and have acquisition of a variety of anaerobic and facultative bacteria (Sobel, 2000; Fredricks *et al.*, 2005). *Lactobacillus iners* is a less studied member of the normal vaginal biota, because it does not grow on Rogosa agar that is typically used to isolate lactobacilli (Vásquez *et al*., 2002). Cultures of vaginal fluid from subjects with BV would typically yield *Gardnerella vaginalis* and a mixture of other bacteria that could include *Prevotella, Porphyromonas, Mobiluncus* and *Mycoplasma* species. It is not known whether BV is primarily initiated by the loss of key lactobacilli or acquisition of the complex bacterial communities found in this syndrome; these may be simultaneous processes (Srinivasan and Fredricks, 2008)

Figure 1.1.

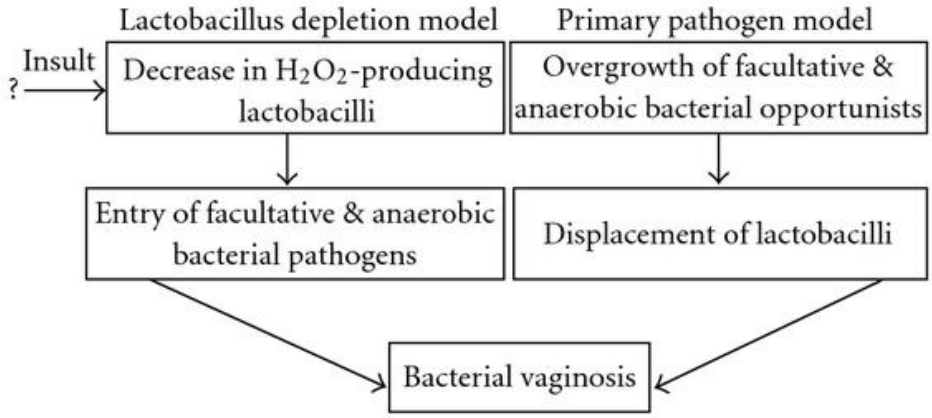


Figure 1.1: Competing models for the pathogenesis of bacterial vaginosis (BV) (Srinivasan and Fredricks, 2008)

At least two models exist to explain the pathogenesis of BV. The lactobacillus depletion model suggests that there is a decrease in hydrogen peroxide producing lactobacilli as the primary event that allows for the overgrowth of facultative anaerobes resulting in BV. The primary pathogen model suggests that the entry of facultative anaerobes causes the displacement of lactobacilli thereby resulting in BV.

It is also likely that some other factor is the primary etiological agent, and that the changes in vaginal microbiota reflect a downstream event in the pathogenesis of BV (Sweet, 2000). Reduction of vaginal lactobacilli has also been associated with douching, use of spermicides and post treatment for other infections with antibiotics (Heng *et al*., 2010; Hooton *et al*., 1991). Natural causes such as lysogenic bacteriophages are also likely causes of a decrease in vaginal lactobacilli (Kiliç *et al*., 2001). BV is associated with an increased risk of pelvic inflammatory disease (PID), postoperative infection, cervicitis, human immunodeficiency virus (HIV), and cervical intraepithelial neoplasia (CIN). Other obstetrical conditions associated with BV include pre-term labour and delivery, low birth weight, premature rupture of membranes, chorioamnionitis (Minkoff *et al*., 1984; McDonald *et al*., 1991; Hillier *et al*., 1995; Hillier and Homes, 1999; Ferris *et al*., 2004) and postpartum endometritis (Sweet, 2000).

1.1.2 Epidemiology of Bacterial Vaginosis

Prevalence of BV varies widely because of differing diagnostic criteria and the population studied (Goldenberg *et al*., 1996). For instant on population, prevalence varies widely with ethnicity from a low prevalence of 6.1% in Asians to 8.8% in Caucasian women, 15.9% in Hispanics and 22.7% African-American women (Goldenberg *et al*., 1996). According to the United States of America (USA) 2001-2004 National Health and Nutrition Survey Data, the prevalence of BV in the USA is 30% in people seeking health care and over 50% in a population of female injection drug users (Allsworth and Peipert, 2007). Among women infected with Human

Immunodeficiency Virus (HIV) attending an HIV out-patient clinic in New Orleans in the USA from January 2002 to January 2004 the prevalence of BV was 49.3% (Kissinger *et al*., 2005). Prevalence of BV in other studies of BV among HIV positive patients ranges from 35% to 47% (Cu-Uvin *et al*., 1999; Jamieson *et al*., 2001; Warren *et al*., 2001). In Kenya, prevalence of BV among women aged 18 to 45 years of age complaining of lower genital-tract-symptoms attending family planning and Sexually Transmitted Disease (STD) clinics in Nairobi was 44% (Bukusi *et al*., 2006). Examination of 1,938 young women entering the military service in the United States of America revealed BV in 27%; the prevalence in sexually experienced participants was 28% compared with 18% in those who had never had sexual intercourse (Yen *et al*., 2003). The epidemiology of BV in many studies suggests a sexually transmissible agent, but this does not explain the high prevalence of BV in sexually inactive women (Livengood, 2009). Several studies have treated the male partners of women with BV with clindamycin and the nitroimidazole agents typically used for the treatment of BV. These studies all failed to demonstrate a decrease in recurrent BV among the women whose partners were treated (Vejtorp *et al*., 1988; Vutyavanich *et al*., 1993; Colli *et al*., 1997). Thus, if BV is caused by a transmissible agent, it is unlikely to be a clindamycin- or nitroimidazole-susceptible anaerobe (Livengood, 2009). In the study on young women entering the military, bacterial vaginosis prevalence was 11% in Asian/Pacific Islanders, which was lower than in other nonwhite ethnic groups ($P = 0.004$) (Yen *et al*., 2003). Clinically, bacterial vaginosis was directly related to multiple sexual partners ($P = 0.026$), self-report of vaginal discharge ($P = 0.001$), self-report of vaginal odor ($P < 0.001$), and concurrent *Chlamydia trachomatis*

infection (P = 0.002), and inversely related to hormonal contraceptive use (P = .013) (Yen *et al.*, 2003). The reason for apparently increased risk of BV among African-American women and the protective effect offered by oral contraceptives remains unclear (Livengood, 2009).

1.1.3 Morbidities Associated with Bacterial Vaginosis

1.1.3.1 BV association with HIV

Symptoms of bacterial vaginosis are troubling enough and notwithstanding they are not the only concern for affected patients (Martin *et al*., 1999). Chief among the other concerns is enhanced susceptibility to STIs and human immunodeficiency virus (HIV) (Martin *et al.*, 1999). Studies have found a significant association between BV and HIV infection (Sewankambo *et al*., 1997; Taha *et al*., 1998; Martin *et al*., 1999; Royce *et al*., 1999). An investigation in North Carolina, U.S.A. confirmed bacterial vaginosis is associated with HIV infection in pregnant women (Royce *et al*., 1999). This was evidenced by comparison of women with normal vaginal flora, where the relative risk for prevalence of HIV infection with intermediate flora was 1.5 (95% confidence interval [CI], 0.2, 12.9) and with abnormal flora was 4.0 (95% CI, 1.1, 14.9) (Royce *et al.* 1999). On the contrary, the association between abnormal vaginal flora and HIV infection could not be explained by age, ethnicity, number of sexual partners in the past six months, sexually transmitted diseases (STDs), or douching during pregnancy (Royce *et al*., 1999).

In a prospective study of Kenyan sex workers, the absence of lactobacilli in vaginal cultures was associated with a 2.0-fold increase in HIV acquisition and a 1.7-fold greater risk of developing gonorrhea (Martin *et al*., 1999). Another similar study in Malawi, Africa found that BV was associated with a 3.7-fold increase in HIV acquisition over a two and half year period (Taha *et al*., 1998). Further, HIV-infected women were reported to shed more viruses into the cervicovaginal discharge when BV was present (Cu-Uvin *et al*., 1999).

1.1.3.2 BV association with other disease conditions

Women with BV were reported to have increased susceptibility to trichomoniasis, chlamydial cervicitis and herpes simplex II infections (Wiesenfeld *et al*., 2003; Cherpes *et al*., 2003). In addition, bacterial vaginosis was found to be a strong predictor of gonorrhea and chlamydial infection among subjects who reported recent exposure to a male partner with urethritis (Wiesenfeld *et al*., 2003).

Bacterial vaginosis appears to enhance the risk of infection after pelvic surgery. Cuff cellulitis following abdominal hysterectomy occurred 3.2 times more often in women with BV in a study of 161 women and in 35% (7/20) of those with preoperative BV (Soper *et al*., 1990). In another study only 8% (4/50) ($P < .1$) of those without BV had cuff cellulitis as compared to 35% with clue cells developing vaginal cuff infections (Larsson *et al*., 1991). Patients with either bacterial vaginosis or trichomoniasis vaginitis were more likely than control subjects to have cuff cellulitis, cuff abscess, or both (relative risk 3.2, 95% confidence interval 1.5 to 6.7 for

bacterial vaginosis; relative risk 3.4, 95% confidence interval 1.6 to 7.1 for trichomoniasis vaginitis) (Soper *et al*., 1990). Pelvic inflammatory disease (PID) after first-trimester pregnancy termination by dilation and curettage procedure (D&C) has been associated with BV (Larsson *et al*., 1992). Another study of postabortal endometritis in 429 women found that preoperative clue cells in the vaginal wet prep was associated with a 5.6-fold (95% CI, 1.8–17.2) increased risk of infection (Hamark and Forssman, 1991).

BV has also been associated with decreased success of *in vitro* fertilization (IVF) procedures and increased risk of cystitis (Eckert *et al*., 2003; Harmanli *et al*., 2000). Patients with BV and with a decreased vaginal log concentration of hydrogen peroxide-producing lactobacilli have decreased conception rates and increased rates of early pregnancy loss after IVF procedure (Eckert *et al*., 2003). In women with bacterial vaginosis, intermediate flora and normal flora, the conception rates were 30% (3/10), 39% (12/31) and 52% (26/50), respectively ($p = 0.06$ for trend). Early pregnancy loss occurred in 33% (1/3), 42% (5/12) and 31% (8/26) of women, respectively ($p = 0.06$, comparing intermediate and normal flora) (Eckert *et al*., 2003).

Many studies have found an association between BV during pregnancy and all major adverse pregnancy outcomes (preterm labor, preterm delivery, low birth weight, premature rupture of membranes, postpartum metritis, intra-amniotic infection) (Livengood, 2009).

1.1.4 Vaginal Flora in Normal versus Bacterial Vaginosis

1.1.4.1 Normal Vaginal Flora

The healthy vaginal flora is dominated by *Lactobacillus* species that produce hydrogen peroxide (**Figure 1.2**); this characteristic eliminates other bacteria unable to synthesize catalase, thus, affording the lactobacilli a tremendous advantage (Livengood, 2009).

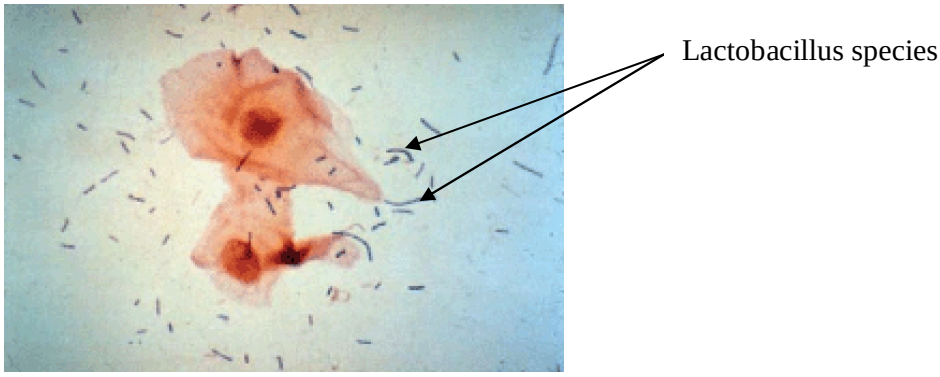


Figure 1.2: Gram stain of normal vaginal contents (original magnification, ×400).
Note predominance of Lactobacillus species that produce hydrogen peroxide, organic acids, and bacteriocins that suppress growth of other species

Desirable vaginal lactobacilli are also powerful organic acid producers. They provide the normal vaginal pH of less than 4.7 using glycogen in the vaginal epithelium as the substrate (Valore *et al*., 2006). They also synthesize bacteriocins which are proteins that inhibit other bacterial species (Valore *et al*., 2006). The power of these lactobacilli to dominate their environment has been reported where exponentially growing *Escherichia coli* were incubated for two hours in vaginal fluid from healthy women and women with BV. The normal fluid caused a 100-fold decline in the *E coli* population, whereas the BV fluid allowed an almost 10-fold increase (Valore *et al.*, 2006).

Although other facultative and anaerobic bacteria, many of which are known pathogens, are always found in the healthy vagina, they are present only in low colony counts (Livengood, 2009). In organotypic cultures of human vaginal epithelium containing dendritic cells, treatment with *Lactobacillus jensenii*, a typical vaginal resident, induced the synthesis of Inter-Leukin-8 mRNA and the epithelial human β-defensin-2 mRNA, but a typical bacterial vaginosis pathogen, *Gardnerella vaginalis*, had no effect. This demonstrates that *Lactobacillus jensenii* has immunostimulatory effect (Valore *et al*., 2006).

Using broad range bacterial PCR with 16S rRNA gene primers 338f and 1407r, 1327 clones were sequenced from 13 subjects without BV (**Figure 1.3**). Of the 1327 clones analyzed, 65.4% of the sequences were *Lactobacillus crispatus* and 28.8% represented *Lactobacillus iners* clones. The remaining 5.8% of clones included other bacteria such as *Gardnerella vaginalis* and other lactobacilli (**Figure 1.3**). These data further validate that subjects without BV have vaginal bacterial biotas dominated by lactobacilli (Srinivasan and Fredricks, 2008).

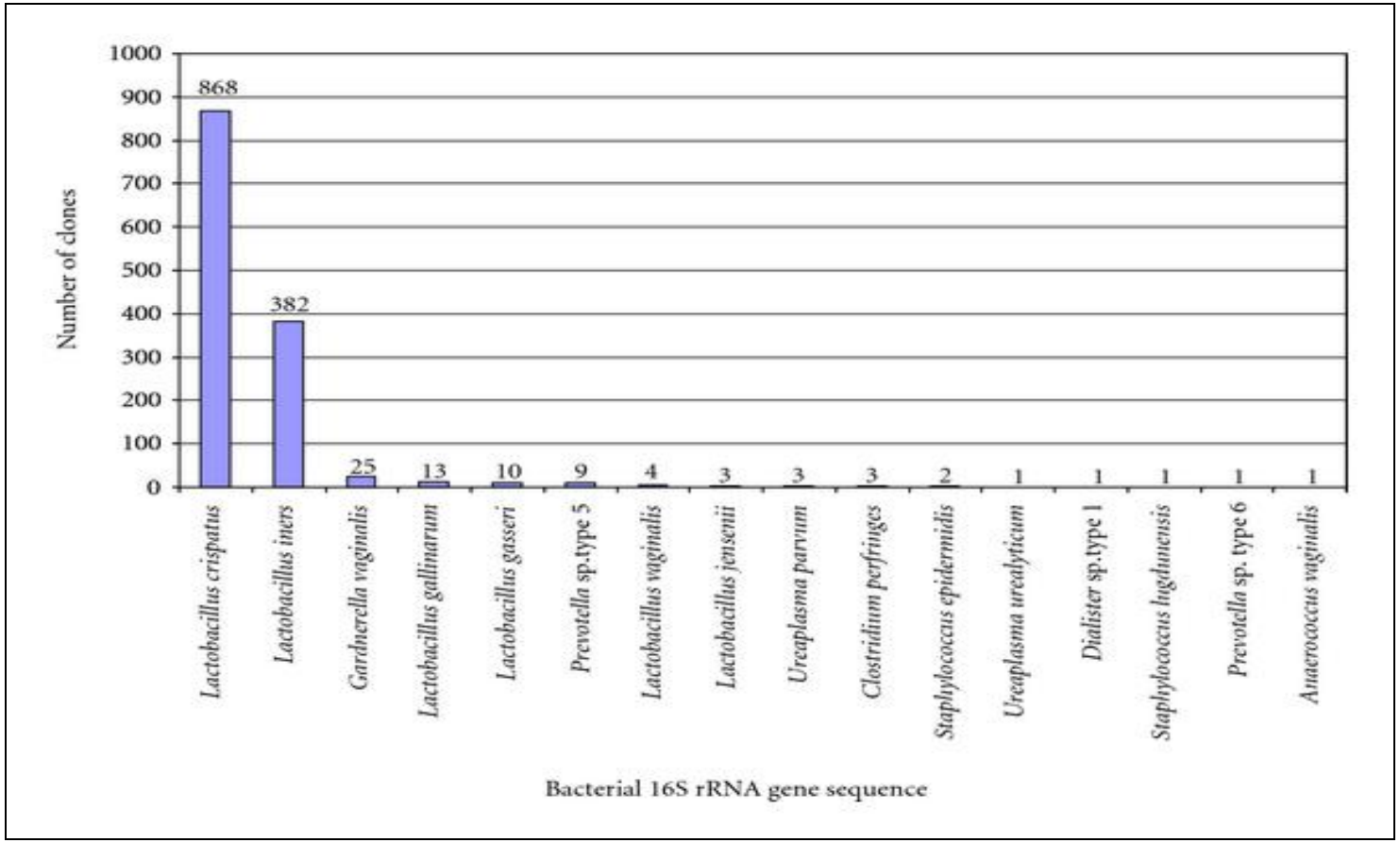


Figure 1.3: *Summary data of rank* abundance plots depicting the bacterial species detected in clone libraries from subjects without BV. Broad range PCR using primers 338f and 1407r along with clone library analysis *of 1327 clones from 13 subjects* without BV resulted in 16 phylotypes being detected. (Srinivasan and Fredricks, 2008).

1.1.4.2 Vaginal Flora in Bacterial Vaginosis

1.1.4.2.1 Gardnerella vaginalis

Gardnerella vaginalis is a gram-variable bacillus (**Figure 1.4**) that was first described in 1953 (Leopold, 1953).

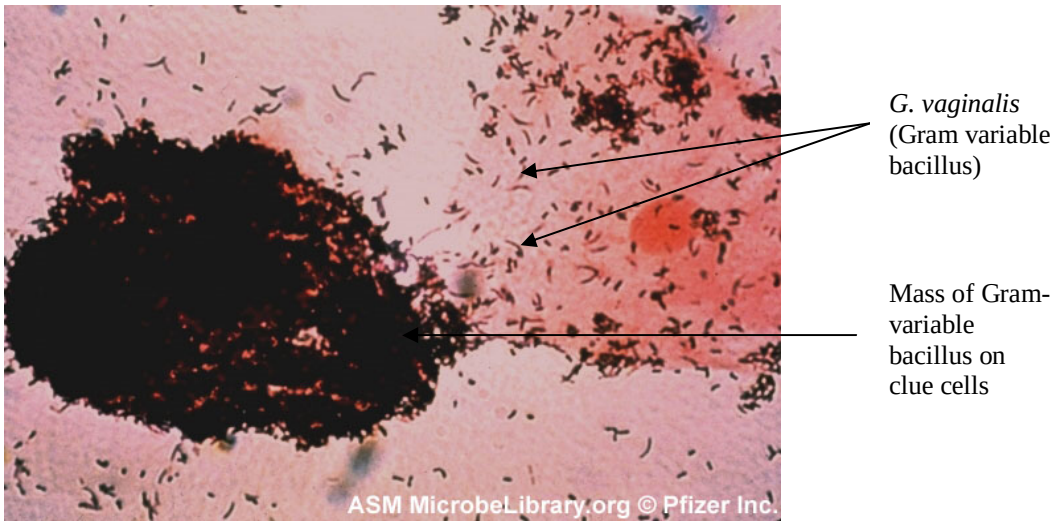


Figure 1.4: Gram stain of *Gardnerella vaginalis*
(from http://lib.jiangnan.edu.cn/ASM/028-2.jpg)

It has also been implicated as the predominant organism in bacterial vaginosis (Gardner and Dukes, 1955). The name *Haemophilus vaginalis* was first proposed by Gardner and Dukes because of the organism's colonial morphology and biochemical profile (Gardner and Dukes, 1955). Because its morphology resembled diphtheroid bacilli in gram-stained preparations, it was subsequently named *Corynebacterium vaginale* (Zinnemann and Turner, 1963). However, because of its variable reaction on Gram staining, being neither typically positive nor typically negative, it was subsequently placed in a unique genus called *Gardnerella* (Greenwood and Pickett, 1980). Its other characteristics

supporting its placement into the new genus are: they are rod-shaped bacteria with laminated cell walls and do not produce catalase and oxidase (Moss and Dunkelberg, 1969). They are facultatively anaerobic and produce acetic acid as their major end product of fermentation (Moss and Dunkelberg, 1969).

The pleomorphic, gram-negative to gram-variable rods average 0.5 by 1.5 pm with no viscible filaments, are non-encapsulated and nonmotile (Greenwood *et al.*, 1977). On vaginalis agar colonies are 0.4 to 0.5 mm in diameter after 48-h incubation at 35°C in a humidified atmosphere with 5% CO_2. The colonies are round, opaque, and smooth (Greenwood *et al*., 1977). *G. vaginalis* is identified using the following biochemical tests (**Table 1.1**).

Table 1.1: Bichemical characteristics of *G. vaginalis*

Substrate	**Acid production**
Dextrin, fructose, galactose, glucose, maltose, mannose, ribose, and starch.	Positive
Arbutin, cellobiose, inositol, mannitol, rhamnose, or sorbitol	Negative
Lactose, sucrose, and xylose	Variable

Adopted from: (Greenwood and Pickett, 1979)**.**

G. vaginalis are fastidious in their growth requirements but do not need nicotinamide adenine dinucleotide (V factor), hemin (X factor), or coenzyme-like substances (Dunkelberg and McVeigh, 1969)**.** They have also been reported to require biotin, folic

acid, niacin, thiamine, riboflavin, and two or more purines-pyrimidines (Dunkelberg and McVeigh, 1969).

Moncla and Pryke (2009) have identified eight different biotypes of *G. vaginalis.* Biotypes 1–4 produce lipase and were reported to be associated with BV though the association of these biotypes with BV is under dispute. Sialidase activity is an important feature in bacterial vaginosis and previous reports have noted the enzyme in 10% of the isolates (von Nicolai *et al*., 1984) and higher rates of 39% have been reported recently though the significance is difficult to access since only 31 strains were examined (Moncla and Pryke, 2009). The association of *G. vaginalis*, sialidase, BV and increases in HIV acquisition rates among women with BV cannot be ruled out (Moncla and Pryke, 2009). In the present study the organism was used as one of the indicators of BV presence.

1.1.4.2.2 Mobiluncus

Mobiluncus are vibrio-like organisms that were observed in vaginal fluids as early as 1895 (Kronig, 1895) and were first isolated from the female genital tract by Curtis in 1913 (Curtis, 1913). These curved motile rod-shaped bacteria continued to be of interest because of their association with bacterial vaginosis (formerly known as nonspecific vaginitis) (Spiegel and Robert, 1984). Although in Spiegel and Robert (1984) study, the Gram reactions of the curved rod-shaped organisms were variable, electron micrographs revealed multilayered gram-positive cell walls lacking an outer membrane. The

apparent absence of an outer membrane suggests that the curved rod-shaped organisms more closely resemble gram-positive organisms than gram-negative organisms (Spiegel and Robert, 1984). This conclusion is supported by reports that these organisms are resistant to colistin and nalidixic acid and are susceptible to penicillin and vancomycin (Sprott *et al*., 1983; Spiegel and Robert, 1984).

Based on DNA homology a new genus, *Mobiluncus*, was proposed for the vaginal isolates. *Mobiluncus* gen. nov. *Mobiluncus* (mo.bi.lun'cus. L. adj. *mobilis* capable of movement, active; L. masc. n. *uncus* a hook; N.L. masc. n. *Mobiluncus* a motile curved rod) (Spiegel and Robert, 1984). In the proposed genus cells are anaerobic, gram variable or gram negative, curved, nonsporing, and rod shaped with tapered ends, occurring singly or in pairs with a gullwing appearance (**Figure 1.5**) (Spiegel and Robert, 1984).

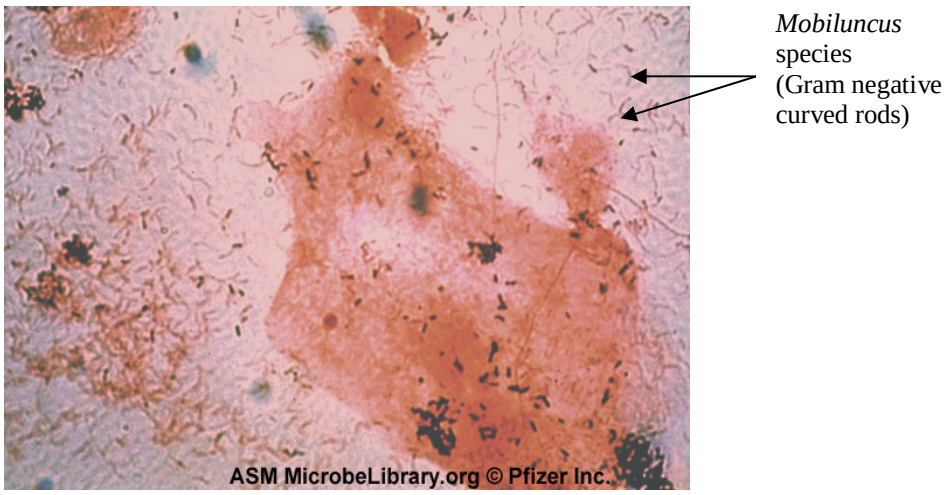


Figure 1.5: Gram stain of *Mobiluncus*.
(from http://lib.jiangnan.edu.cn/ASM/028-3.jpg)

Mobiluncus are motile by means of multiple subpolar flagella and possess a multilayered gram-positive type of cell wall. Weakly (terminal pH**,** 5.5 to 6.5) or strongly (terminal pH, 5.5) saccharolytic. Fermentation products include succinic and acetic acids, with or without lactic acid (Spiegel and Robert, 1984). Growth is stimulated by rabbit serum (Spiegel and Robert, 1984). Growth is not stimulated by formate-fumarate. Oxidase and catalase are not produced. The described species are found in human vaginae associated with bacterial vaginosis (nonspecific vaginitis). The type species is *Mobiluncus curtisii. Mobiluncus curtisii* subsp. *curtisii* sp. nov. *Mobiluncus curtisii* subsp. *curtisii* (cur.ti'si.i. N.L. gen. n. *curtisii* named after A. H. Curtis, who isolated the first strain) (Spiegel and Robert, 1984).

Multivariate analysis of vaginal flora has shown that *Mobiluncus* spp. occur in large numbers in bacterial vaginosis (BV) and may therefore serve as an indicator organism for this clinical syndrome (Hillier *et al*., 1991; Bahar *et al*., 2005). *Mobiluncus* species have been used as an indicator for BV because of their frequent isolation from vaginal smears of affected patients (Roberts *et al*., 1985; Hillier *et al*., 1991; Bahar *et al*., 2005).

The curved anaerobic bacilli have shown a greater degree of adherence to epithelial cells than does *Gardnerella vaginalis*, which suggests that these curved bacilli can be responsible for the presence of clue cells (Fredricsson *et al*., 1984). In the current study presence of *Mobiluncus* species was screened.

1.1.4.2.3 Bacteroides

Bacteroides species are anaerobic, bile-resistant, non-spore-forming, gram-negative rods (**Figure 1.6)** and may be either motile or non-motile, depending on the species (Liu *et al.*, 2003; Madigan, 2005).

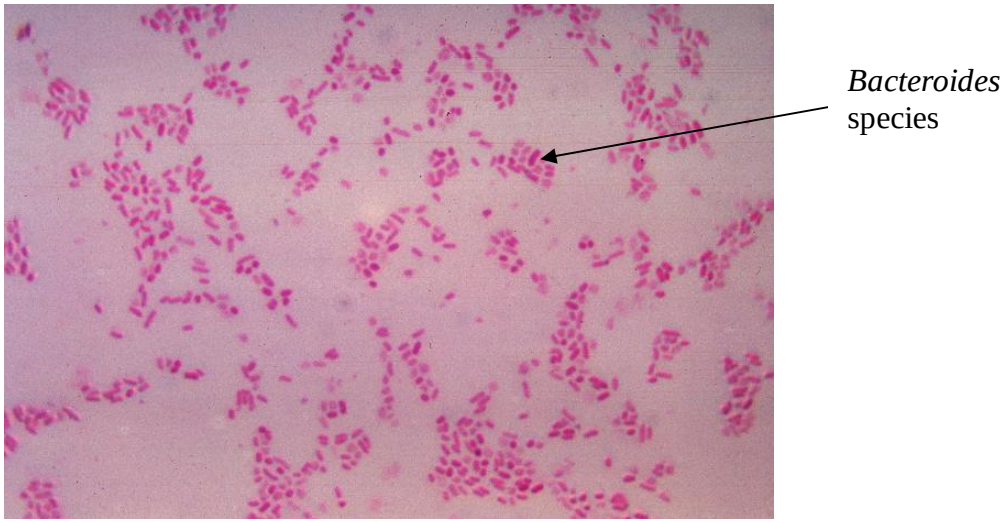


Figure 1.6: Gram stain of *Bacteroides*
(http://en.academic.ru/dic.nsf/enwiki/2119445)

The taxonomy of *Bacteroides* has undergone major revisions in the last few decades, but the genus is now limited to species within the *Bacteroides fragilis* group, which now number greater than 20 members (Liu *et al.*, 2003). The DNA base composition is 40-48% GC. Unusual to bacteria, *Bacteroides* membranes contain sphingolipids (Madigan, 2005). They also contain meso-diaminopimelic acid in their peptidoglycan layer (Madigan, 2005). The presence of *Bacteroides* in the vagina was first reported in 1928 where *B. melaninogenicus* was isolated from 28 out of 35 normal women (Burdon,

1928) and has recently been reclassified and split into *Prevotella melaninogenica* and *Prevotella intermedia* (Brook, 2008). Bacteroides species are significant clinical pathogens and are found in most anaerobic infections, with an associated mortality of more than 19%. The bacteria maintain a complex and generally beneficial relationship with the host when retained in the gut, but when they escape this environment they can cause significant pathology (Wexler, 2007). The rates of vaginal carriage of *Bacteroides* in both healthy pregnant and nonpregnant women have been estimated to be between 0 and 6% (Lindner *et al*., 1978; Leszczynski *et al*., 1997), but higher rates of 16% have been reported in women in labor (Leszczyński *et al*., 1995) and highest rates of 27 to 28% in patients with cervicitis (Lindner *et al*., 1978).

Prevotella (Bacteroides) species and *Bacteroides fragilis* have also been associated with preterm birth (Minkoff *et al*., 1984; McDonald *et al*., 1991; Krohn *et al*., 1991). The mechanism by which lower genital tract infection causes upper genital tract disease resulting to preterm birth remains poorly understood although sialidases (neuraminidases) which are enzymes which enhance the ability of microorganisms to invade and destroy tissue have been implicated (Moncla *et al*., 1990). In a study by Briselden *et al.,* (1992) elevated levels of sialidase activity were detected in 42 (84%) of 50 vaginal fluid specimens from women with bacterial vaginosis and none of 19 vaginal fluids from women without bacterial vaginosis (P less than 0.001). To determine the probable source of sialidases in the vaginal fluid, the microorganisms recovered from women with bacterial vaginosis before and after treatment were assayed (Briselden *et al*., 1992). Of 28 specimens from women with bacterial vaginosis, 27 (96%) yielded

sialidase-positive bacteria (Briselden *et al.*, 1992). *Prevotella* and *Bacteroides* species accounted for the sialidase activity in 26 of the vaginal fluids, and *Gardnerella vaginalis* accounted for the sialidase activity in the remaining fluid (Briselden *et al*., 1992). *Bacteroides fragilis* is thus a significant organism in BV and was thus used as an indicator of BV presence in present study.

1.1.4.2.4 Atopobium

Based on comparative sequence analyses of sequences obtained by 16S rRNA gene sequencing of *Lactobacillus minutus, Lactobacillus rimae* and *Streptococcus parvulus* a new genus *Atopobium* gen. nov., was proposed by Collins and Wallbanks (1992). The organisms represented an unknown line of descent within the lactic acid group of bacteria (Collins and Wallbanks, 1992).

Phylogenetically, *Atopobium* species form a distinct grouping which forms a node with the actinomycete line of descent (Stackebrandt and Ludwig, 1994). The closest phylogenetic relative of the genus *Atopobium* is *Coriobacterium glomerans*, an isolate from the intestinal tract of red soldier bugs (Pyrrhocoris apterus) (Haas and Konig, 1988).

Atopobium species consist of anaerobic, Gram positive elliptical cocci or rod-shaped (**Figure 1.7**) organisms which produce large amounts of lactic acid (Ferris *et al.*, 2004). On this basis, some species belonging to the genus *Atopobium* were originally identified

as *Lactobacillus sp*. (Ferris *et al*., 2004). Despite their phylogenetic association within the actinomycete branch of the Gram-positive bacteria, *Atopobium* species are not phenotypically exceptional and consequently they are sometimes confused with the low G+C content lactic acid group of bacteria (Rodriguez Jovita *et al*., 1999).

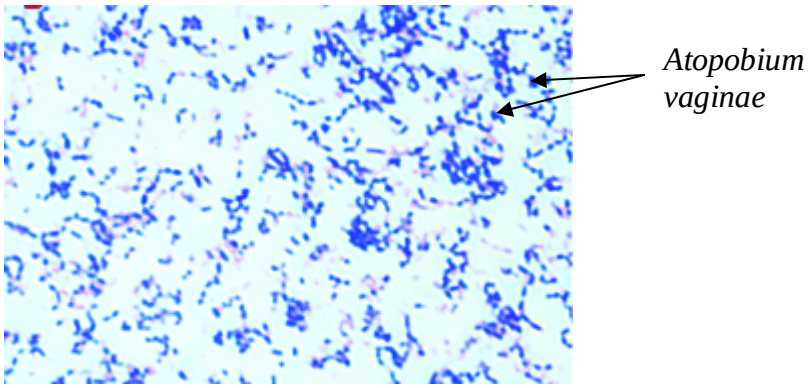


Figure 1.7: Gram stain of *Atopobium vaginae*.
(from: http://jcm.asm.org/cgi/content/full/41/6/2788/F1)

Organisms previously identified as *G. morbillorum* by the API ID32A system have been later identified as *A. vaginae* using molecular techniques (Geissdörfer *et al*., 2003; Ferris *et al*., 2004). It is not surprising that *A. vaginae* has not been previously recognized in women with BV due to its Gram stain appearance of a small Gram positive cocco-bacillus and members of the genus being known to produce large amounts of lactic acid (Ferris *et al*., 2004).

The association of *A. vaginae* with abnormal vaginal flora has recently been reported (Ferris *et al*., 2004). The appearance of prominent *A. vaginae* bands in 55% of patients with Nugent scores of greater than 3 and the presence of *A. vaginae* DGGE bands in

only 8.3% of samples from patients with normal flora is an indicator that the species are prominent in patients with abnormal vaginal secretions (Ferris *et al*., 2004).

Ferris *et al.*, (2004) further explains that "it is unlikely that the frequency of the *A. vaginae* bands in BV-positive samples, and their absence from normal samples, is an artifact of the PCR amplification/denaturing gradient gel electrophoresis (DGGE) method. The PCR primers complement sites on both *Lactobacillus* spp. and *A. vaginae* 16S rRNA genes, and *A. vaginae* and *Lactobacillus* bands co-occur in some specimens (**Figure 1.8**). Thus there is no evidence that primer bias toward *Lactobacillus spp*. would explain the lack of *A. vaginae* bands in DGGE patterns of normal patients. The appearance of prominent *A. vaginae* bands in DGGE patterns of BV-positive patients is likely a true reflection of their prevalence in the bacterial ecology that constitutes the BV syndrome".

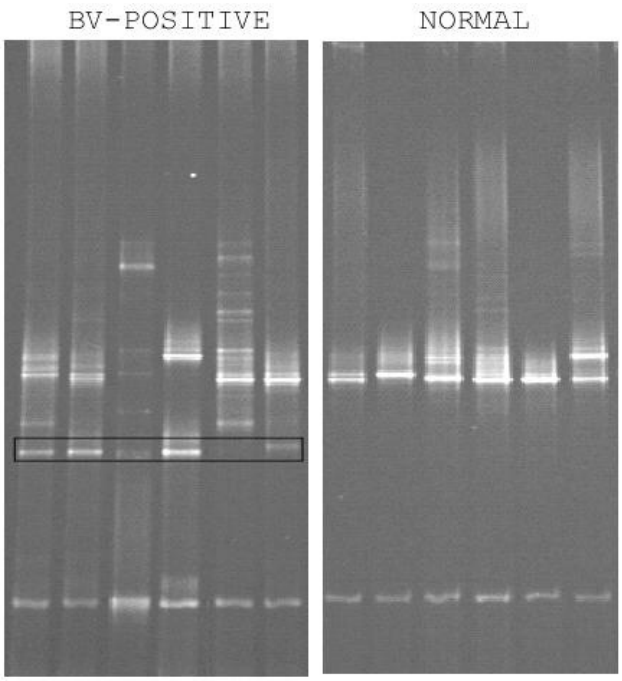


Figure 1.8: Normal vs. BV positive DGGE banding patterns
Examples of denaturing gradient gel electrophoresis banding patterns obtained from 16S rRNA gene segments PCR-amplified from nucleic acids isolated from vaginal lavage samples of patients clinically described as having BV-positive or normal vaginal flora. The boxed area encloses examples of *A. vaginae* bands. These were found in 55% of BV-positive samples (Ferris *et al.*, 2004)**.**

1.1.4.2.5 Other Micro-organisms in BV Flora

Broad range bacterial PCR in combination with cloning and sequencing presents a reasonable option to estimate the diversity of the most abundant bacteria associated with BV but is an expensive approach with low throughput (Srinivasan and Fredricks, 2008).

In a study by Thies *et al.* (2007) bacterial communities in 50 women with BV as determined by Nugent scoring were characterized using a combination of broad range PCR amplification of the 16S rRNA gene, in combination with T-RFLP fingerprinting

and a total of 23 bacteria phylotypes were detected in the samples. The phylotopes included *Atopobium vaginae*, *Gardnerella vaginalis*, *Megasphaera* sp., *Lactobacillus iners*, *Eggerthella* sp. and BVAB1 (Bacterial Vaginosis Associated Bacterium), BVAB2, and BVAB3 (Thies *et al*., 2007). Other phylotypes have also been identified (**Figure 1.9**) (Srinivasan and Fredricks, 2008) and another study has shown higher bacterial diversity in subjects who had bacterial vaginosis, where 35 bacterial phylotypes were detected (Fredricks *et al*., 2005b). Sixteen of the bacterial species detected seemed to be newly recognized on the basis of poor homology with known 16S rDNA sequences in GenBank (Fredricks *et al*., 2005; Fredricks *et al*., 2007).

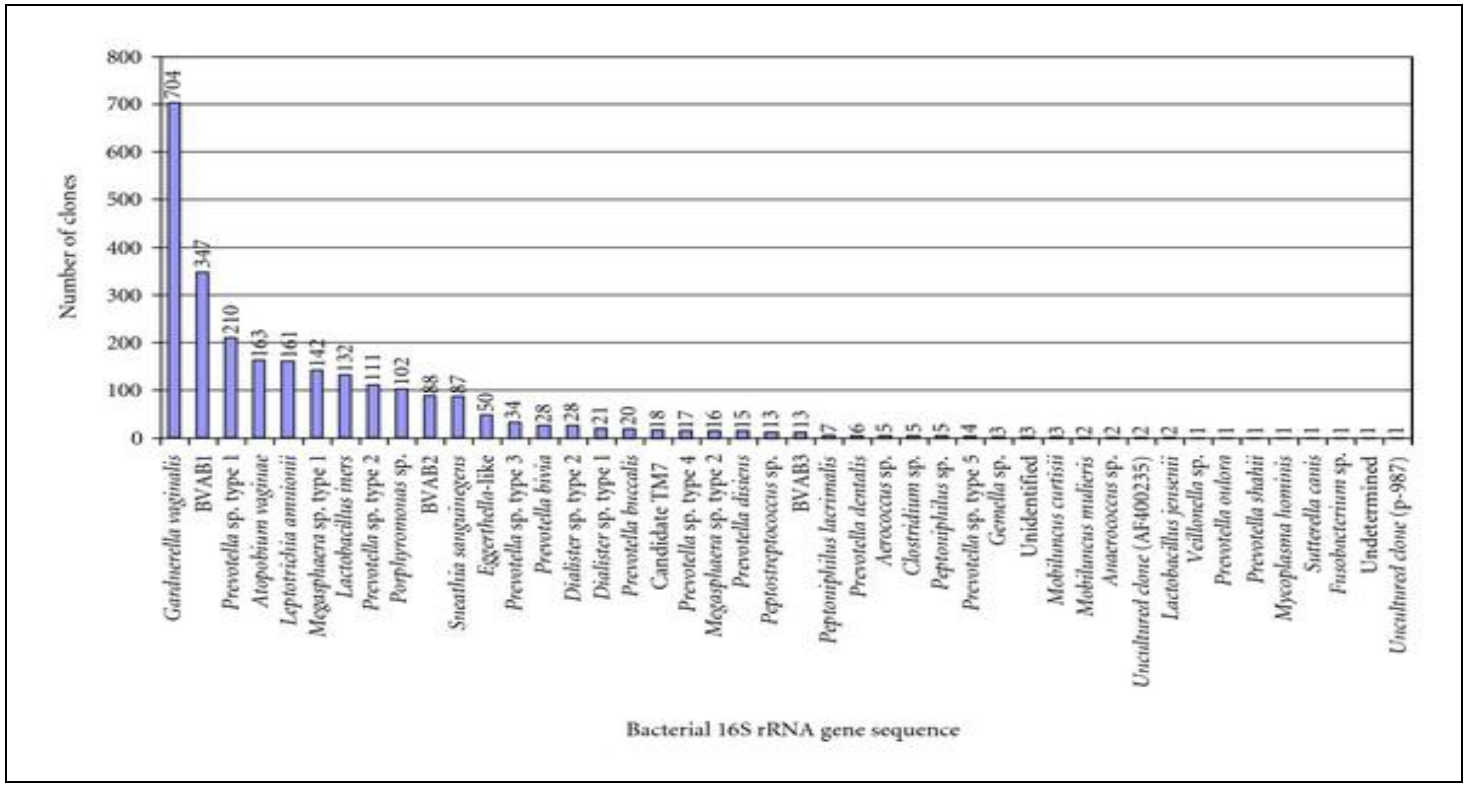


Figure 1.9: Summary data of rank abundance plots depicting the bacterial species detected in clone libraries from subjects with BV
Broad range PCR using primers 338f and 1407r along with clone library analysis of 2577 clones from 23 clone libraries from 17 subjects with BV resulted in the detection of 44 different bacterial species. Vaginal bacterial species are indicated on the x-axis and the numbers of clones are indicated on the y-axis and above every bar. BVAB denotes bacterial vaginosis associated bacterium (Srinivasan and Fredricks, 2008).

With increasing build up of microorganisms associated with BV it may be an example of a condition produced by a pathogenic microbial community rather than a single pathogen (Srinivasan and Fredricks, 2008). This presents many challenges for understanding the etiology and pathogenesis of this syndrome (Srinivasan and Fredricks, 2008).

Bifidobacterium involvement with BV is contradictory as different studies report different results. A study in pregnant women had *Bifidobacterium* present in 94% of BV positive, 58% of intermediate BV and 12% of healthy controls (Rosenstein *et al*., 1996). In contrast, a different study reported the importance of *Bifidobacteria* species as a probiotic in correction of vaginal flora (Korshunov *et al*., 1999). Therefore, the role of *Bifidobacterium* in BV requires further investigation.

1.1.5 Bacteriophages and Bacterial Vaginosis

The description of possible association of vaginal lactobacilli lysogenic phage with BV development in 2001 (Kiliç *et al*., 2001) is quite recent compared to 1935 Whitehead and Cox (1935) when the detrimental effects of phage infection in lactobacilli used in milk industries was reported. Despite the fact that an earlier description of lactobacilli phage in milk industries was reported, over 70 years later phages remain the largest single cause of fermentation failure in the dairy industry (Kiliç *et al*., 2001).

Generally bacteriophages can be divided into two groups: virulent phages capable of only lytic (**Figure 1.10**) propagation and temperate phages (**Figure 1.11**) capable of either lytic or lysogenic propagation (Oki *et al.*, 1996).

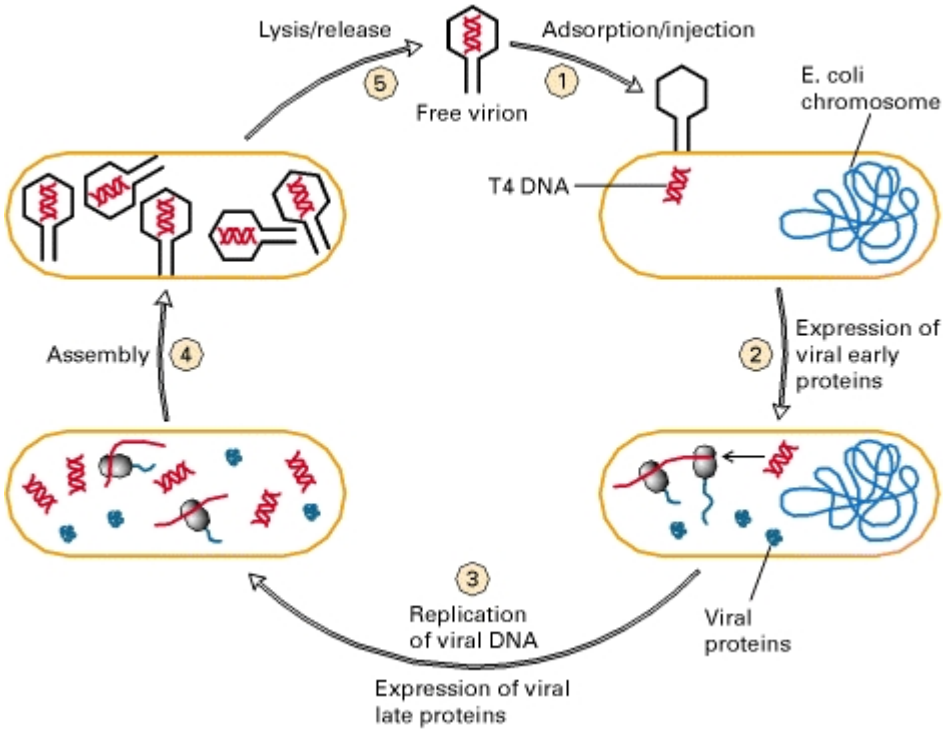


Figure 1.10: The steps in the lytic replication cycle of a nonenveloped virus are illustrated for *E. coli* bacteriophage T4
During adsorption (step **1**), viral coat proteins (at the tip of the tail in T4) interact with specific receptor proteins on the exterior of the host cell. The viral genome is then injected into the host. Next, host-cell enzymes transcribe viral "early" genes into mRNAs and subsequently translate these into viral "early" proteins (step **2**), which replicate the viral DNA and induce expression of viral "late" proteins by host-cell enzymes (step **3**). The viral late proteins include capsid and assembly proteins and enzymes that degrade the host-cell DNA, supplying nucleotides for synthesis of viral DNA. Progeny virions are assembled in the cell (step **4**) and released (step **5**) when the cell is lysed by viral proteins. Newly liberated viruses initiate another cycle of infection in other host cells

(From: http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=mcb&part=A1408)

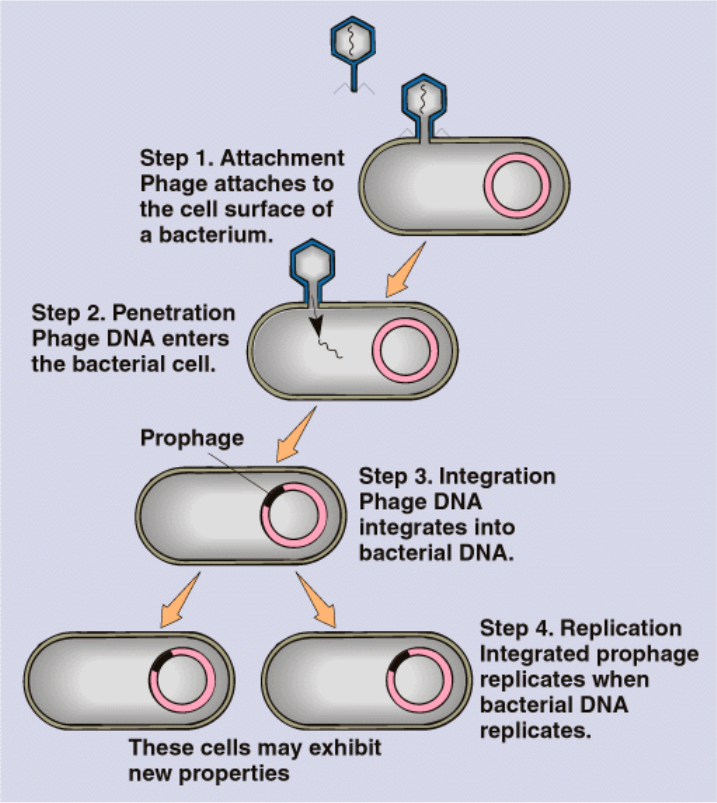


Figure 1.11: Lyfe Cycle of temperate phage (Lysogenic)
(From: http://www.zo.utexas.edu/faculty/sjasper/images/so23_03.gif)

The division into the two groups is based on their end effect on the infected bacteria. Hence, there is a possibility that vaginal *Lactobacillus* are lysed by lytic phage while on the other hand it could be by a lysogenic phage with lytic properties (Oki *et al*., 1996). For release of phage particles from the host cell, lysogenic phages appear to encode a set of enzymes, which degrade the host cell envelope and the lysis genes seem to be expressed under a complicated control (Oki *et al*., 1996). In several phages, the cytolytic process has been presumed to depend upon phage-encoded holins and lysins proteins. Several presumptive lysins and holins have been reported from phages of lactic

acid bacteria (Oki *et al*., 1996). Using mitomysin C, vaginal lactobacilli were induced to release phage and Bradley type A and B phages (**Figure 1.12)** were observed by electron microscopy from the formed plaques. It was hence, concluded that phage may play a role in reduction of *Lactobacillus* and that more studies need to be done to determine phage association with BV development (Kiliç *et al*., 2001).

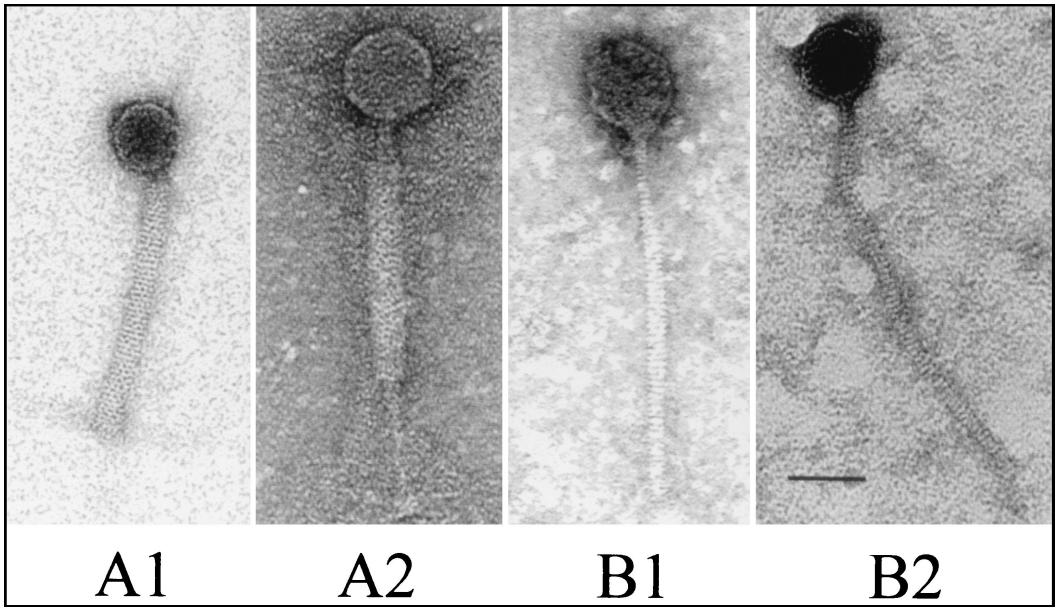


Figure 1.12: Electron micrograph of vaginal *Lactobacillus* phages. A1, fkc21T; A2, fkc12a; B1, fkc39; B2, fkc7a. Bar 5 50 nm. (Kiliç *et al*., 2001).

Smoking has been shown to increases a woman's risk of contracting bacterial vaginosis (BV) (Hillier *et al*., 1995). Studies on chemicals contained in cigarette smoke have shown that trace amounts of benzo[a]pyrene diol epoxide (BPDE) can be found in vaginal secretion of women who smoke (Pavlova and Tao, 2000). To determine the role of BPDE in reducing lactobacilli in the vagina *in vitro* studies performed have demonstrated the ability of the chemical to significantly increase phage induction in

Lactobacillus. This finding implies that smoking may reduce vaginal lactobacilli by promoting phage induction (Pavlova and Tao, 2000).

Bearing in mind that phage can confer protection to the host bacteria as described by (Wagner and Waldor, 2002) then the mitomysin C induced lysogenic phage isolated in (Kiliç *et al*., 2001) study may be beneficial to the lactobacilli. Determination of the presence of phage types A and B was done in the present study.

1.1.6 Diagnosis of Bacterial Vaginosis

Presently no method for diagnosing BV can be regarded as the best (Forsum *et al*., 2005). It could be partly based on inferred knowledge on the part of the clinical investigators scoring in the clinic – various scoring systems have been chosen which fit to a particular BV related problem in a particular population (Forsum *et al*., 2005). In brief, there are a variety of methods used for diagnosis of BV. None of the methods identifies a particular microbe as the etiological agent of BV; rather, the methods are all based on the criteria diagnostic principles (Forsum *et al*., 2005).

1.1.6.1 Symptoms

The main symptoms associated with Bacterial vaginosis are; firstly, vaginal discharge that is normally a thin whitish, grayish or yellowish discharge from the vagina with a cloudy look, secondly, fishy vaginal odour - this is one of the more unpleasant symptoms. Thirdly, vaginal odour after sexual intercourse has a much strong unpleasant vaginal odour, and fourthly, vaginal itching and painful intercourse are not so common

but are still associated with this disorder (Klebanoff *et al.*, 2004). In the case for most women however, no symptoms are manifest and the condition goes unnoticed (Klebanoff *et al*., 2004).

1.1.6.2 Specimen sampling

Vaginal swabs or cervicovaginal lavage (CVLs) specimen are both appropriate for diagnosis of BV (Kissinger *et al.*, 2005). In a study on a population of HIV-infected women, specimen collection by CVL was equivalent for BV and superior for TV when compared with collection by swab using objective gold standards (Kissinger *et al*., 2005). Therefore, CVL may serve as a good alternative to swabs for researchers and clinicians alike since comparable sensitivities suggest that CVL can be used for detection of BV with equal reliability as wet preparation. The study therefore, provides evidence that specimen collection by CVL is similar to specimen collection by vaginal swab for BV diagnosis (Kissinger *et al*., 2005).

1.1.6.3 Diagnosis using Amsel Criteria

Clinical diagnosis of BV is based on the presence of three of the following four Amsel criteria: that is firstly, an adherent grayish-white discharge, secondly, an elevated vaginal pH ($\geq$ 4.5), thirdly, fishy odour on addition of 10% KOH to the discharge (positive sniff/whiff test), and lastly, the presence of clue cells on wet mount of vaginal specimen (Amsel *et al.*, 1983). The fishy odour is due to presence of amines in the vaginal fluid that become volatile at increased pH. Trimethylamine is likely to be the

source of odour though putrescene and cadeverine may also be present (Chen *et al*., 1979).

1.1.6.4 Diagnosis using Nugent criteria

Gram stain - based Nugent diagnostic criteria is the Gold standard for the diagnosis of BV (Nugent *et al*., 1991). In this criterion the vaginal flora can be categorized as normal that is *Lactobacillus* predominant, intermediate that is with mixed flora, or bacterial vaginosis (Nugent *et al*., 1991). In BV, *Lactobacillus* species morphotypes are diminished or undetectable microscopically and are replaced by large numbers of small Gram variable (*G. vaginalis*) and small Gram negative short rods including *Bacteroides* species, *Prevotella* species, *Porphyromonas* species with or without curved Gram variable rods (*Mobiluncus species*). Gram-positive cocci in chains and fusiform Gram-negative rods are occasionally observed (Nugent *et al*., 1991).

To provide a more standardized method of interpreting Gram stain a scoring system was developed. The bacterial morphology are quantified under oil immersion field (OIF) as 0 (none) seen, 1+ ($\leq$1/OIF) seen, 2+ (1 – 5/OIF) seen, 3+ (6 – 30/OIF) seen and 4+ (>30/OIF) seen and a score assigned to each of the three groups The score of each of the three morphotypes is summed and a score of '0 – 3' is interpreted as normal, '4 – 6' as 'intermediate'/altered vaginal flora (AVF) and '7 – 10' as BV (Nugent *et al*., 1991).

The Nugent method therefore does not permit the identification of several bacteria implicated in BV such as *Mycoplasma* species (lack a cell wall) and *Atopobium vaginae* (presents variable morphology) (Ferris *et al*., 2004; Verhelst *et al*., 2004). Though, well suited for use in resource-poor settings, it requires well-trained, highly experienced individuals to interpret the results (Dumonceaux *et al*., 2009). According to Nugent criteria patients in the intermediate and the normal categories are generally not considered for treatment (Nugent *et al*., 1991). Therefore, any laboratory test that can distinguish between bacterial vaginosis from nonbacterial vaginosis should be adequate for management purposes (Obata-Yasuoka *et al*., 2002).

1.1.6.5 Proline aminopeptidase activity

The activity of proline aminopeptidase has been determined in a study on vaginal wet preparations from 57 patients and compared it to Spiegel Gram's stain method (Schoonmaker *et al*., 1991). Values for samples identified as bacterial vaginosis positive were significantly different ($p < 0.0001$) from those that were negative according to the Spiegel analysis of Gram's stain: negative results, 66 +/- 41 mU/ml; positive results, 704 +/-145 mU/ml. The findings indicate that proline aminopeptidase assay may offer a rapid, sensitive, and objective laboratory method for the diagnosis of bacterial vaginosis (Schoonmaker *et al*., 1991). The findings have however, never been confirmed in other reports in peer-reviewed journals (Forsum *et al*., 2005).

1.1.6.6 Electronic sensor array "electronic nose"

The idea of using sensor arrays coupled with software interpretation of the resulting signals ("electronic nose") for the diagnosis of BV is based on the assumption that the signal pattern thus detected might be an electronic counterpart to the human sensory sensation of smell (Forsum *et al.*, 2005).

In a small pilot study AromaScan technology was found to be of possible value in screening for bacterial vaginosis, particularly in settings where assessing the clinical criteria objectively may be difficult (Chandiok *et al.*, 1997). Once the equipment is set up, the test is quick and simple, and gives an easy to understand answer. The results of using AromaScan are so far generally disappointing and the potential application of the technology for diagnosis of other clinical conditions associated with a characteristic odour should be assessed (Chandiok *et al.*, 1997).

Osmetech Microbial Analyser (OMA) working on the same principle of “electronic nose” has also been evaluated (Hay *et al.*, 2003). The sensitivity and specificity of the OMA were 81.45% and 76.1% compared to Amsel criteria and 82.9% and 77.3% compared to Gramstain. Further refinements to improve the sensitivity and specificity of the OMA are required to provide an accurate near patient testing method (Hay *et al.*, 2003).

1.1.6.7 Diagnosis by Multiplex PCR

Several molecular techniques have been reported for diagnosis of BV which involves PCR detection of organisms associated with the condition (Menard *et al.*, 2008). A recently reported method uses molecular quantification of *Gardnerella vaginalis* and *Atopobium vaginae* loads as an indicator of BV presence (Menard *et al*., 2008).

Obata-Yasuoka *et al.,* (2002) developed a multiplex PCR based diagnostic method for BV. Their method differentiates bacterial vaginosis from nonbacterial vaginosis using multiple primers designed from sequences of anaerobes that are implicated in bacterial vaginosis. The primers used in the multiplex PCR were designed to amplify the 16S ribosomal DNA from *Mobilancus mulieris* and *Mobiluncus curtisii*, the gene encoding neuraminidase (nanH) from *Bacteroides fragilis*, and an internal spacer region of ribosomal DNA from *Gardnerella vaginalis* (Obata-Yasuoka *et al*., 2002). Their choice of organism to screen for is based on the fact that *M. mulieris, M. curtisii, B. fragilis,* and *G. vaginalis* are the most common anaerobes associated with bacterial vaginosis, and that their predominance in the vaginal flora is indicative of bacterial vaginosis. In this study multiplex PCR was used for screening BV positive specimen.

1.1.6.8 New advents in BV diagnosis

Most bacteria shown to have strong associations with BV represent bacteria that have been detected by cultivation and identified by traditional morphological and biochemical

methods (Ferris *et al*., 2004). Surveys of microbes in environmental samples using molecular techniques have constantly shown that cultivated species do not represent the full complement of microbes in most habitats (Hugenholtz *et al*., 1998). The use of molecular methods such as microarrays and shot gun approaches of sequencing are now becoming common in survey of bacterial flora in mammalian systems (Kazor *et al*., 2003).

DNA microarrays are useful in a large number of applications especially where high-throughput is needed (Dufva, 2009). DNA microarray has the capacity to probe a sample for hundred to million different molecules at once. Microarray technology is a complex mixture of numerous technology and research fields such as mechanics, microfabrication, chemistry, DNA behaviour, microfluidics, enzymology, optics and bioinformatics (Dufva, 2009). DNA microarray testing has recently emerged as a new technology that is promising for broad-spectrum virus detection (Wang *et al*., 2002; Lin *et al*., 2007). An in-house microarray platform has previously been designed to detect all known viruses as well as novel viruses related to known viral families (Virochip; University of California San Francisco [UCSF]) (Wang *et al*., 2002).

The Virochip comprises of 22, 000 oligonucleotide probes representing all 1800 fully or partially sequenced viruses (including phages) in GenBank as of Fall 2004 (Chiu *et al*., 2008). The Virochip performance in detection of viruses has been tested previously using virally infected tissue culture cells and in selected patient cohorts (Kistler *et al*., 2007; Chiu *et al*., 2008). The ViroChip made its bona fide first appearance when

scientists were trying to figure out what was causing the Severe Acute Respiratory Syndrome (SARS) outbreak in 2003 (http://www.genomenewsnetwork.org/articles/2004/10/01/virochip.php). The U.S.A. Centers for Disease Control and Prevention shipped a sample to DeRisi's laboratory (http://derisilab.ucsf.edu/) in the Department of Biochemistry, UCSF and within 24 hours they had used the ViroChip to characterize the virus as a novel coronavirus (Prof. Joseph DeRisi, personal communication, January, 2006). The Virochip was used in this study for detection of phages and viruses in BV positive and BV negative specimen. In concideration that the ViroChip is designed to detect all known viruses as well as novel viruses related to known viral families (Wang *et al.*, 2002); the present study, also used shot gun sequencing technique which has been demonstrated to be a powerful tool for sequencing of completely novel organisms (Anderson, 1981; Fleischmann *et al.*, 1995).

In shot gun sequencing a large DNA fragment can be completely sequenced by cloning smaller subfragments in a single-stranded phage vector to produce a representative library, then sequencing randomly-chosen clones using the dideoxynucleotide chain termination method and a flanking universal primer (Anderson, 1981; Sanger *et al.*, 1992; Chan, 2005). This method, of building the final sequence as a composite of overlapping subfragment sequences, has been aptly termed "shotgun" DNA sequencing (Anderson, 1981; Chan, 2005).

The shot gun method was first applied to obtain the complete nucleotide sequence (1,830,137 base pairs) of the genome from the bacterium *Haemophilus influenzae* Rd (Fleischmann *et al*., 1995). The approach does not require the initial mapping efforts and is thus applicable to the vast array of microbial species for which genome maps are unavailable (Fleischmann *et al*., 1995). It is for this reason that the current study uses shot gun sequencing in addition to micro-array with the aim of identifying any known or novel organisms that may be involved in development of BV.

1.1.7 Treatment and Management of Bacterial Vaginosis

Accurate diagnosis of BV, as well as the explanation of effective prevention and treatment strategies, remains a major challenge (Morris *et al*., 2001). Hence, current recommended treatment regimens for BV are based not on the eradication of a known pathogen but on the restoration of a pattern of normal vaginal flora as established historically using culture-based methods (Bradshaw *et al*., 2006). The Centers for Disease Control and Prevention (CDC) have recommended several treatment regimens for bacterial vaginosis (Workowski and Berman, 2006). The regimen comprises of oral metronidazole, 500 mg twice daily for seven days or 0.75% metronidazole gel, or one full applicator 5g intravaginally, once a day for five days (Livengood *et al*., 1999) or Clindamycin cream, 2%, one full applicator 5g intravaginally at bedtime for seven days (Workowski and Berman, 2006; Chen *et al*., 2009). Patients are advised to avoid consuming alcohol during treatment with metronidazole and for 24 hours thereafter (Workowski and Berman, 2006). Clindamycin cream is oil-based and might weaken

latex condoms and diaphragms for five days after use. Alternative regimen is Clindamycin 300mg orally twice a day for seven days or Clindamycin ovules 100mg intravaginally once at bedtime for three days (Sobel *et al*., 2001; Workowski and Berman, 2006).

Metronidazole has unpleasant side effects including gastrointestinal upset, a metallic taste in the mouth, and skin rash (Bradshaw *et al*., 2006). For these reasons, alternative therapies have been tried as well as lighter regimens of metrodinazole, such as the single 2-g oral dose though has lowest efficacy (Bradshaw *et al*., 2006; Workowski and Berman, 2006). Secnidazole, a 5-nitroimidazole derivate that is structurally related to the commonly used 5-nitroimidazole as metronidazole and tinidazole has also been tried (Núñez and Gómez, 2005). Secnidazole is rapidly and completely absorbed after oral administration, has an average serum half-life of 17 to 28.8 hours, and, in women, has a half-life of 14.3±1.3 hours. A single 1g oral dose of secnidazole has been found to be effective in treatment of BV associated to *G. vaginalis* (Núñez and Gómez, 2005).

Use of probiotics specifically designed for women's health have been proposed for use in BV treatment (Reid *et al*., 2001). The most evidence has come from the work on *L rhamnosus* GR-1 in combination with *L. fermentum* B-54 and RC-14. These organisms are antagonistic to the growth and adhesion of various intestinal and urogenital pathogens including *Salmonella typhimurium, Shigella sonnei, E coli* 0157, toxic shock *Staphylococcus aureus*, group *B streptococci*, *Enterococcus faecalis*, *G vaginalis*, and

uropathogenic *E coli* (Reid *et al*., 2001; Reid *et al*., 2003). Of greater importance, the administration of these lactobacilli by mouth and intravaginally has been shown to be safe and to reduce the risk of UTI, BV and yeast vaginitis. Additional attributes of probiotics include their potential to degrade lipids and enhance cytokine levels, which promote embryo development and thus preventing preterm birth (Reid and Bocking, 2003).

Other strains are being investigated to be administered after metronidazole therapy for BV. They include *L. crispatus* CTV05, *L. crispatus* and *Lactobacillus salivarius* strains and strains with elevated arginine deaminase activity which are believed to have a greater therapeutic potential than strains producing only hydrogen peroxide (Famularo *et al*., 2001; Ocaña and Nader-Macías, 2001).

1.1.8 Relapse of Bacterial Vaginosis

Since the pathogenesis of BV is not known, there is no clear distinction between re-infection and relapse (Sobel, 1997). Recurrent BV is common with 15 – 30% of women having symptomatic BV recurring in 30 – 90 days following therapy and 70% recurring within nine months (Brotman *et al*., 2007). It is therefore, essential to learn how to prevent relapse/incidence of BV in order to help the symptomatic women who experience considerable distress from recurrent BV, as well as aiming for a potential public health impact to reduce BV associated adverse pregnancy outcomes and HIV transmission (Hay, 2005). Despite increased understanding of the factors controlling the

microbial interactions in the vaginal environment there has not yet been a successful intervention to prevent relapse of BV. The use of repeated courses of antibiotics is not an attractive long term proposition since bacterial resistance will develop (Hay, 2005). A more physiological approach would be to use acidification and/or hydrogen peroxide to assist the lactobacilli during menstruation or after unprotected sexual intercourse. There are uncontrolled studies showing that hydrogen peroxide is successful (Cardone *et al.*, 2003).

Prospective studies that further our understanding of the relationship between microbial vaginal ecology and Pelvic Inflammatory Diseases may lead to strategies that prevent the devastating consequences (Brotman *et al*., 2007). This is only achievable if a specific etiological agent can be identified and thus enabling targeted treatement.

1.2 Problem statement

Bacterial vaginosis occurs in women of reproductive age and affects both HIV and non HIV infected. The prevalence of BV among HIV infected women ranges from 35% to 49% while in non HIV infected it ranges from 6% to 23% based on different studies in widely varied populations. This indicates that the prevalence of BV is much higher in women infected with HIV and the largest populations of HIV infections are in Africa. The prevalence of BV is thus much higher in Africa. The condition has adverse effects associated with it which include increased risk of pelvic inflammatory disease (PID), postoperative infection, cervicitis, human immunodeficiency virus (HIV), cervical

intraepithelial neoplasia (CIN), preterm labour and delivery, low birth weight, premature rupture of membranes and chorioamnionitis. Despite all these effects the etiology of BV remains unknown and attempts to identify the cause by many studies are based on culture methods. Advanced molecular studies on environmental ecosystems have shown that majority of bacteria in most environments are unculturable. It is equally possible that the etiology of BV remains unknown because a non culturable bacterium is involved. Unfortunately advanced molecular techniques are expensive and unaffordable in African countries with Kenya included. To bridge the gap simpler molecular tools need to be designed for use in developing countries. This can be achieved by collaboration with developing countries where advanced tools are available.

1.3 Justification of the Present Study

Understanding the etiology of BV would be helpful in the control or prevention of the prevalent condition in both HIV infected and non HIV infected. In order to make use of advanced molecular tools that will detect both culturable and non culturable microorganisms a study with two phases was designed. The first phase was a pilot study done in an advanced laboratory so as to utilize advanced non culture based molecular techniques in identification of organisms associated with BV. Development of simpler tools was also possible and important in that phase of the study. Use of advanced tools that can also detect novel organisms in phase one study would be a significant step forward from previous attempts that use culture methods. Unique organisms associated with presence or abscence of BV identified in the first phase would then be screened in a

larger population in a developing country using the simple and affordable tools developed. In the second phase it was therefore, important to determine the role that the significant bacteria identified in the first phase have in the development of BV in a larger sample size. A different study had identified *Lactobacillus* bacteriophages that may be playing a role in BV development. It was therefore, important to determine the prevalence of the identified bacteriophages in the two study phases of the present work. In addition, with prevalence of BV being higher among HIV infected women, use of samples from HIV infected women had higher possibilities of obtaining sufficient BV positive samples for sufficient comparison with BV negative samples. The findings of the two phased study would therefore provide an avenue for discovery of known and novel unculturable as well as culturable microorganisms involved in BV development. This will improve the management and prevention of BV in both HIV and non HIV infected women.

1.4 Hypothesis

The following hypothesis was explored in this study:

1. Specific novel and unculturable microorganisms cause the development of bacterial vaginosis in women infected with HIV.

1.5 Objectives

1.5.1 Main Objective

1. To determine the novel and predominant unculturable micro-organisms associated with presence or absence of bacterial vaginosis in women infected with HIV.

1.5.2 Specific Objectives

1. To determine the novel and predominant unculturable bacteria associated with BV in Californian women infected with HIV
2. To determine the relationship between the four Bradley types of *Lactobacillus* bacteriophages and bacterial vaginosis
3. To determine the prevalence and the pattern of change in BV status with time progression among HIV positive women in Kenya
4. To determine the association of the identified novel and predominant unculturable bacteria with BV status among HIV positive women in Kenya

CHAPTER TWO

2.0 Materials and Methods

2.1 Study site

The study was carried out in two phases at two different sites. The first phase was a pilot study which was carried out at, the Department of Biochemistry, University of California, San Francisco (USA) and the second phase at Centre for Microbiology Research/Welcome Trust Research Laboratory at Kenya Medical Research Institute (KEMRI) in Kenya.

2.2 Study design

Two study designs were used; cross sectional design for the study done in the University of California, San Francisco and retrospective longitudinal study design for the phase done in KEMRI.

2.3 Specimen source and sampling

For the pilot phase of the study in UCSF, the vaginal swabs already obtained for the clinical diagnosis of BV were resuspended in glass tubes containing 3ml sterile phosphate buffered saline and transported to the Laboratory at the Biochemistry Department in a cool box at 4 ^{o}C. The participants were recruited at the Women's Health-Mount Zion Clinic. The women were members of the long-standing Women's Interagency HIV Study (WIHS) cohort. No patient information was obtained, and no identifiers were placed on the specimen tube. Only the date the samples were collected

and the BV microscopy screening results were used for this study. Ethical approval to carry out the study was obtained from the Institution Review Board of the University of California, San Francisco (Project number 06030018).

The cervicovaginal lavage (CVL) specimens used in the study at KEMRI were obtained through collaboration with the Centre for Respiratory Disease Research, KEMRI. The CVL specimens were previously collected for the study on "Natural history of HIV-1 infection and steroid hormone contraception - SSC No: 515" and stored frozen at -70^{o}C in the Centre for Microbiology Research, KEMRI. The CVLs were collected from a cohort of 330 HIV-1-infected women every six months for a period of up to four years between the years 2003 to 2006. The samples had been collected after the patients willingly agreed to participate in the study after the consent explanation and had signed a consent agreement (see **Appendix 1).** Signed consent to collect the salt water wash (CVLs), store and use them for future research had been obtained. No personal information was obtained from the patient's records and the samples were de-identified and coded differently to eliminate any possibility of being linked to any patient's identity. The only information that was retained was the date of samples collection so as to provide progressive data to enable observation of trend with time.

2.3.1 Inclusion Criteria of Samples

2.3.1.1 Pilot study in California

Vaginal swabs collected from HIV positive women on antiretroviral therapy.

Vaginal swabs collected from HIV positive women who complied with antiretroviral therapy usage.

Vaginal swabs collected during non-menstrual period (without blood).

Vaginal swabs collected for diagnosis of BV.

Vaginal swabs collected from women who were members of the long-standing Women's Interagency HIV Study (WIHS) cohort.

2.3.1.2 Retrospective Study in Kenya

Cervicovaginal lavage collected from HIV positive women on antiretroviral therapy.

Cervicovaginal lavage collected from HIV positive women who complied with antiretroviral therapy usage.

Cervicovaginal lavage collected during non-menstrual period (without blood).

Cervicovaginal lavage collected from women whose CD4 counts were ≥500 cells/µl.

Cervicovaginal lavage collected from sexually active women aged 18 to 40 years.

Cervicovaginal lavage collected from women who complied with the 6 months visit interval.

Cervicovaginal lavage collected from women who had made 4 or more visits uninterrupted.

2.3.2 Exclusion Criteria of Samples

2.3.2.1 Pilot study in California

Vaginal swabs collected from HIV positive women not on antiretroviral therapy.

Vaginal swabs collected from HIV positive women who did not comply with antiretroviral therapy usage.

Vaginal swabs collected during menstrual period (with blood in sample).

Vaginal swabs collected for diagnosis of other infections other than BV.

Vaginal swabs collected from women who were not members of the long-standing Women's Interagency HIV Study (WIHS) cohort.

2.3.2.2 Retrospective Study in Kenya

Cervicovaginal lavage collected from HIV positive women not on antiretroviral therapy.

Cervicovaginal lavage collected from HIV positive women who did not comply with antiretroviral therapy usage.

Cervicovaginal lavage collected during menstrual period (with blood in sample).

Cervicovaginal lavage collected from women whose CD4 counts were $\leq$500 cells/µl or not known.

Cervicovaginal lavage collected from sexually inactive women aged over 40 years.

Cervicovaginal lavage collected from women who did not comply with the 6 months visit interval.

Cervicovaginal lavage collected from women who had made less than 4 visits.

2.4 Laboratory Procedures

2.4.1 Isolation of DNA from vaginal swabs

The tube containing the vaginal swab was vortexed and the swab discarded under sterile conditions. A volume of 3 ml of the PBS solution was filtered through a 0.45 µm pore filter. The filtrate was then transferred into SW-55 ultracentrifuge tubes and treated with RNAse and DNAse for 30min at 37^{o}C. The tubes were then centrifuged at 35,000 rpm for 1 hour 5 minutes. The supernatant was discarded and the pellet re-suspended in 200 µl Tris HCl pH 8.0. The solution was transferred into 1.5 ml microfuge tubes and 5µl of 10% SDS added. The tubes were then incubated at 68^{o}C for 5 minutes followed by phenol-chloroform extraction of DNA. Into the tube 200 µl of buffered phenol (Sigma) was added and the tube vortexed for ten minutes. Centrifugation was done for 10 minutes in a microfuge at 13,000 rpm. The aqueous top layer was removed and transfered to a fresh microfuge tube. The phenol extraction step was repeated and 200 µl of chloroform was added and vortexed briefly. The microtube was centrifuged for five minutes and the aqueous top layer transferred to a fresh tube. Chloroform extraction step was repeated and 20 µl 3 M sodium acetate pH 4.8 was added to the tube. Two volumes of ice cold 100% ethanol was added to the tube and centrifuged for ten minutes at 13,000 rpm in a 4 ^{o}C refrigerated centrifuge. Ethanol was poured off and 1ml of 100% ethanol added. The tube was centrifuged for 5 minutes, ethanol poured off and the pellet dried under a vacuum after which a small white pellet was viscible. The pellet was re-suspended in 100 µl of micro-pore filter sterilized distilled water. The DNA was

quantified on a nanodrop spectrophotometer and stored in -20 ^{o}C.

2.4.2 Random Amplification of DNA from vaginal swabs

This procedure was to randomly amplify any given sample of DNA with as much representation as possible. It is not a "linear" method, but is useful to compare relative enrichment between two samples. This protocol has been used successfully to amplify genomic representations of less than 1 ng of DNA (Bohlander *et al*., 1992; Wang *et al*., 2002). Two sets of enzymatic reactions were used: round A and round B. In both reactions water was included as a negative control template.

2.4.2.1 Round A amplification

Sequenase was used to extend randomly annealed primer A to generate templates for subsequent PCR during Round B. The reaction mixture comprised of sequenase (13 units/µl), 5X sequenase buffer, sequenase dilution buffer, 3 mM dNTP mix, 500ug/ml BSA, 0.1 M DTT, 40 pmol/µl Primer A: GTT TCC CAG TCA CGA TCN NNN NNN NN and DNA template. The cycling procedure of round A amplification was ramp from 10 °C to 37 °C over 8 min. Hold at 37 °C for 8 min; rapid ramp to 94 °C and hold for 2 min. Rapid ramp to 10°C and hold for 5 min at 10 °C while adding 1.2 µl of diluted Sequenase (1:4 dilution). Ramp from 10 °C to 37 °C over 8 min. Hold at 37°C for 8 min (**Appendix 2**). The product was then used for Round B amplification.

2.4.2.2 Round B amplification

During Round B, the specific primer B was used to amplify the templates previously generated round A. The mixture comprised of 10X PCR Buffer (500 mM KCl, 100 mM Tris pH 8.3), 25 mM $MgCl_2$, 100X dNTPs (20 mM each nucleotide), 5 unit/µl Taq polymerase and 100 pmol/µl Primer B: GTT TCC CAG TCA CGA TC in a 50 µl reaction. The PCR cycles used were 35 cycles of 94 ℃ for 30 seconds, 40 ℃ for 30 seconds, 50 ℃ for 30 seconds followed by one cycle of 72 ℃ for 2 minutes (**Appendix 2**).

25 µl of the PCR products from Round B amplification were resolved by electrophoresis at 115V DC on a 2% agarose gel. The bands were visualized on UV illumination and the bands cut out from the gel and the DNA extracted using a QIAquick Gel Extraction Kit® (Qiagen, USA) in accordance with the manufactures instructions. The purified DNA was used for construction of DNA library and sequencing. Round B PCR product (5-10 µl) was used as a template for Round C PCR reaction. The procedure was the same as Round B only that aminoallyl dNTPs were used (**Appendix 2**). The product was used for micro array experiments.

2.4.3 Micro array experiments

2.4.3.1 Printing and Post Processing of Micro Array Slides

Individual 70-mer viral oligos designed from every completely sequenced virus from GenBank were printed on to poly-l-lysine coated slides using a lab designed robot. The oligos were designed based on a taxon-by-taxon approach.

Before use of the slides post processing was done to bind the DNA more tightly to the slide, excess DNA that had not bound, was removed and any free lysines on the poly-lysine slide coating blocked (those free lysines are "sticky" and if not blocked they could non-specifically bind labeled probe during an array hybridization) (**Appendix 3**). Since slides age more quickly after they have been post-processed, only the batch to be used at a time was post-processed.

2.4.3.2 Dye Coupling and Hybridization

The product of Round C amplification reaction was cleaned using Zymo DNA Clean and Concentrator™-5 kit as follows. In a separate 1.5 ml tube 200 µl of DNA binding buffer was mixed with the PCR product from Round C. The mixture was placed into Zymo column and was centrifuged for 2 minutes at 4000 rpm. This step was repeated once. To the column 200 µl of wash buffer was added and was centrifuged for 30 seconds at 13,000 rpm. After a repeat centrifugation for 1.5 minutes the column was eluted into a new 1.5 ml tube by adding 9.5 µl of water

The zymo cleaned sample and probe 70 were coupled with cy3 and cy5 dyes, respectively. The cy3 and cy5 dyes were dissolved in 6 µl DMSO. To the sample 1 µl of 1M bicarbonate solution and 1 µl of cy3 dye were added. Probe 70 mixture was prepared by adding 1µl bicarbonate, 1 µl cy5, 1 µl probe 70 (1 picogram/µl) and water added to make a total volume to 10 µl. The mixtures were then incubated in the dark for 30 minutes. After incubation the sample and probe 70 mixtures were cleaned with the Zymo kit as earlier described and eluted with 17.5 µl of water.

The sample and probe 70 were mixed in the following order and volumes;

- 16 µl cy5/probe70 mixture
- 16 µl cy3/sample mixture
- 6 µl 20x SSC
- 1 µl 1M Hepes *(extra mixing prior to adding SDS)*
- 1 µl 10% SDS

The mixture was then placed on 100^{o}C block for two minutes, centrifuged, allowed to cool and then loaded on the array. The array was placed in the hybridization chamber base and 10 µl of water added into all the small reservoirs in the base. The chamber lid was then positioned on top and held to the base using sealing screws by applying downward pressure and turning the screws in a clockwise manner until turning becomes difficult (3-4 half turns) (**Appendix 4**). After checking that the rubber sealing gasket is

seated correctly in the gasket groove, the hybridization chamber was incubated in a 65ºC water bath overnight.

2.4.3.3 Post-hybridisation Processing of Micro Array Slides

After incubation, the hybridization chamber was removed from the water bath and the outside dried by blotting with paper towels. The screws were lossened completely and the lid removed. The slide was removed and quickly transferred into a rack and incubated inside a staining dish containing Wash Buffer A consisting of 1X SSC and 0.2% SDS at 25°C for 5 min. Following incubation in buffer A the slide was transferred to a second staining dish containing Wash Buffer B consisting of 0.1X SSC and 0.2% SDS at 25°C for 5 min. The slide was then transferred into a third staining dish containing Wash Buffer C consisting of 0.1X SSC for 30 sec at 25°C to remove trace SDS (**Appendix 4**). The microarray slide was then air dried and scanned on an Axon GenePix 4000a Microarray Scanner with photomultiplicator level (PMT) set at 600. Images were visualized and analysed at wavelengeth ratio 635/532 using GenePix Pro software (**Appendix 5**). GenePix Pro Report files (.gpr) were exported to E-predict software (http://derisilab.ucsf.edu/software/epredict/index.html) for normalization (Urisman *et al*., 2005). E-predict output files (.cdt) were exported to Cluster software (version 2.0) and profiles clustered by hierarchical average linkage clastering with Pearson correlation as the similarity metric. Cluster images were viewed and obtained using Java TreeView software (version 1.0.8). Arrays with intensities below 100 were not considered for further analysis since they do not show distinct profiles on clustering.

An alliquote of the sample used for hybridization was also used for shot gun sequencing, providing a validation of the array results. A sample spiked with hela cells and a known virus was used as a positive control in all the processes including; nucleic acid extraction, PCR amplification and array hybridization.

2.4.4 Cloning of PCR Products

The gel purified PCR product from Round B reaction was used for cloning. Invitrogen TOPO® TA cloning kit was used. A volume of 3 µl of gel purified PCR product was added into 1.5 ml tubes. Into the purified PCR product, 1 µl of salt solution and 1 µl of water were added followed by addition of 1 µl TOPO® Vector and gently mixed. The TOPO® TA reaction mixture was incubated at room temperature for five minutes and then placed in ice. One Shot® Chemical Transformation *E. coli* (Invitrogen, USA) tube was thawed on ice and to it was added 4 µl of the TOPO TA reaction mix and let to stand on ice for 30 minutes. The cells were then heat shocked at 42^{o}C for 45 seconds and the tube transferred to ice immediately. Into the tube containing cells 250 µl of room temperature S.O.C. (Invitrogen, USA) medium was added. The tube was capped and placed into a shaker at 37^{o}C for 1 hour after which 50 µl was spread on pre-warmed LB plates containing X-gal and 50 µg/ml kanamycin. The plates were incubated overnight at 37^{o}C. Ninety five white colonies were picked for sequencing from each tube (sample).

2.4.5 Whole Cell PCR and Big Dye Sequencing Reaction

Standard PCR was done on 96 well PCR plates. Reaction mixture comprised of 10X PCR buffer, 50 mM MgCl2, 25 mM dNTP mix, 5 U/µl rTaq and 20 pmole/µl M13 forward and reverse primers. The products were resolved on 2% agarose gel and cleaned using 75% Isopropanol **(Appendix 6)**. Cleaned products were used as templates for big dye sequencing PCR reaction. The mixture comprised of 5X sequencing reaction buffer, 5pmole/µL single primer and big dye mix (**Appendix 6**). Products of the sequencing reaction were washed and resuspended in HIDI formamide. Heat denaturation was done for one minute at 96^{o}C before loading into the ABI Genetic Analyzer (Applied Biosystems, USA).

2.4.6 Detection of BV associated bacteria in cervicovaginal lavage by multiplex PCR

Diagnosis of BV in the CVL samples was done using the method of Obata-Yasuoka *et al.,* (2002) as described henceforth. The CVL samples in 2 ml screw cup tubes were centrifuged at 14,000 rpm for ten minutes to pellet the bacteria. The supernatant was aspirated and frozen for later isolation of phage and the cell pellet re-suspended in 50 µl of distilled water and boiled for 10 minutes and centrifuged as above. The final supernatant provided the PCR template for multiplex PCR and for other PCR screens. The primers in **Table 2.1** below were used in the multiplex PCR reaction. The specificity of the primers had been determined by sequencing of the PCR products

(Obata-Yasuoka *et al.*, 2002). Normal saline spiked with identified *Mobiluncus*, *Bacteroides* and *Gardnerella* species in 2 ml screw cup tubes was used as a positive control sample.

Table 2.1: Primers used in multiplex PCR detection of bacterial vaginosis associated organisms

Species	Target DNA	Primers	Sequence (5'to 3')	Product size	Reference
Mobiluncus species	16S rDNA	Mob2A Mob2B	GTGAGTAACCTGTCCTTTTCT TTTAAGAGATTAGCCCCACCT	1015 bp	Obata-Yasuoka *et al.*, 2002
Bacteroides fragilis	*nanH**	BFnA BFnB	TTCGCTTTTCTGTTTTCTGTGT CAGCAACCACCCAAACATTATT	842 bp	Obata-Yasuoka *et al.*, 2002
Gardnerella vaginalis	Internal spacer region of rDNA	GarSA GarSB	GGTTTTGCTTGTTTTTACTTTTA GCTACTTGGGAATAAACATAAAA	570 bp	Obata-Yasuoka *et al.*, 2002

DNA = deoxyribonucleic acid; rDNA = ribosomal deoxyribonucleic acid; bp = base pair.
* Gene encoding neuraminidase

The PCR program used comprised of the first 10 cycles of touchdown routine, followed by an additional 30 routine amplification cycles. The touchdown routine had a preliminary denaturation for one minute at 94^{o}C, denaturation for one minute at 94^{o}C, ramping at 1.5^{o}C per second to the 60^{o}C touchdown annealing temperature. The subsequent routine amplification comprised of denaturation for one minute at 94^{o}C, ramping at 1.5^{o}C per second to the annealing temperature, annealing for one minute at 60^{o}C, and extension for one minute at 72^{o}C, with a final extension step of seven minutes at 72^{o}C. The PCR products were resolved by electrophoresis at 115V DC using a 2% agarose gel.

2.4.7 PCR Screening of Bifidobacteria and Oenococcus

Presence of *Bifidobacteria* was screened using published primers that are genus specific. The primers used are shown in **Table 2.2**.

Table 2.2: Primers used for detection of *Bifidobacteria*

Primer	Sequence (5' to 3')	Specificity or target	Product size	Reference
Bif164-f	GGGTGGTAATGCCGGATG	*Bifidobacterium* 16S	520 bp	Langendijk *et al.*,1995
Bif662-r	CCACCGTTACACCGGGAA	*Bifidobacterium* 16S		

The PCR thermocycling program for Bif164-f and Bif662-r primers was: 94°C for five minutes; 35 cycles of 94°C for 30 seconds, 62°C for 20 seconds, and 68°C 40 seconds; 62°C for 20 seconds; and 68°C for seven minutes.

Presence of *Oenococcus oeni* in the CVL samples was screened using primers in **Table 2.3**. The primers are unpublished and were designed from sequences obtained by "shotgun" sequencing done during the pilot study at UCSF. Sequences that had significance sequence similarity with *Oenococcus* or *Leuconostoc* were aligned using the web based ClustalW software (http://www.ebi.ac.uk/Tools/clustalw/, 22/8/2007). The primers were then designed from the conserved region using FastPCR software and analyzed for hair pins and dimmers using integrated DNA technology web based software (http://www.idtdna.com/SciTools/SciTools.aspx, 22/8/2007). PCR reactions were done and the bands obtained sequenced to confirm the specificity of the designed primers. The designed primers shown in **Table 2.3** below were used for screening *Oenococcus* (formerly called *Leuconostoc*).

Table 2.3: Primers used for detection of *Oenococcus*

Primer	Sequence (5' to 3')	Specificity or target	Product size
LeuOR	GAACATATTGGCCTGATTGG	*Leuconostoc/Oenococcus*	1174 bp
LeuOF	TACAGACACAAACATTCAGG	*Leuconostoc/Oenococcus*	

NB: The primers were designed in the present study

2.4.8 Isolation of DNA from vaginal *Lactobacilus* bacteriophages

Stored supernatants from vaginal swabs (California study) and cervicalvaginal fluids (Kenya study) were thawed and equilibrated with the room temperature. They were then filtered through 0.45µm syringe filter and subsequently treated with PEG (7% PEG molecular weight 8000 with 0.5M NaCl) to concentrate the phage particles. The PEG was added and mixed thoroughly and effluents held at 58^{o}C for one hour. The

PEG precipitate was then collected by centrifugation at 27,000 X g for 30 minutes at 58^{o}C. The supernatant was discarded and the pellet re-suspended in 200 µl Tris HCl pH 8.0 followed by addition of 5 µl of 10% SDS. Phenol-chloroform extraction of DNA was done as described in the protocol above for isolation of DNA from vaginal swabs. The clean DNA was recovered by sodium acetate and precipitated with ethanol. The pellet was re-suspended in 20 µl of micro-pore filter sterilized distilled water and used as a template for PCR to detect the phage types A1, A2, B1 and B2. The primers and the PCR protocol used have previously been described (Kiliç *et al.*, 2001).

2.4.9 Identification of vaginal *Lactobacillus* bacteriophages using PCR

To determine the presence of the four different classes of lactobacillus phages previously described as types A1, A2, B1 and B2 the following set of primers were used (Kiliç *et al.*, 2001) (**Table 2.4**).

Table 2.4: Primers used for bacteriophage detection and classification

Type	Strain	Primer	Sequence	Product size
A1	fkc5a	Forward	5'-ATGCTGACGGAAGGTGTGGTCAATGCT-3'	480 bp
		Reverse	5'-AGTGCTACAACAGCCCTTGCACCGT-3'	
A2	fkc12a	Forward	5'-GCGGTTTATCTGGAAGTATAGCCCT-3'	326 bp
		Reverse	5'-CTGATGCCAACCTTCACCATGAAGCCT-3'	
B1	fkc39	Forward	5'-CGAACTGGCGAATTTGTACCATCT-3'	237 bp
		Reverse	5'-GTCGCCAGTTGTTGAAGCAGTGATGT-3'	
B2	fTL76	Forward	5'-CACCTCCGAGTGACATGGGCACAGCT-3'	250 bp
		Reverse	5'-GCAATTGCAAATACTGCACCA-3'	

The four sets of primers were from (Kiliç *et al*., 2001)

The product from Round B PCR reaction was used as the template DNA. The thermal cycling program used was as follows: initial denaturation at 94°C for two minutes and 35 cycles of 94°C for one minute, 50°C for two minutes, and 72°C for three minutes. Finally, there was an extension step at 72°C for seven minutes. The PCR DNA products were analyzed for correct sizes and for purity on 2% agarose gel by electrophoresis at 115V DC.

2.5 Data management and statistical analysis

Experimental data was recorded in MS excel software and Chi square was used to determine any association of data that can be put into tables with mutually exclusive and exhaustive cells. Percentage frequencies were determined and presented in form of graphs and histograms. Prevalence of BV, *Bifidobacteria*, *Oenococcus* and phage in the Kenyan study population was also determined. Analysis of variance (ANOVA) and Kruskal-Wallis Nonparametric ANOVA Test were used as appropriate to determine the variations among means of different possible categories. Graphpad Instat^{tm} V2.04 and Statview © software were used for statistical analysis. Association was considered to be present if there was a significant presence of an organism in BV positive or negative samples. The significance was determined by Chi-squared test of independence

CHAPTER THREE

3.0 Results

3.1 Pilot study in California

A total of twenty six vaginal swabs were collected from Women's Health-Mount Zion Clinic in San Francisco, California. They comprised of twelve BV positive and fourteen BV negative as determined by microscopy using Nugent criteria (Nugent *et al.*, 1991).

3.1.1 Isolation and amplification of DNA from vaginal swabs

The twenty six samples had DNA successfully isolated and the DNA concentrations were greater than 50 ng/µl as measured by the nanodrop. All samples amplified, with products of amplification with primer B resolving as a smear on 2% agarose gel with amplicons ranging between 300bp and 600bp (**Figure 3.1**)

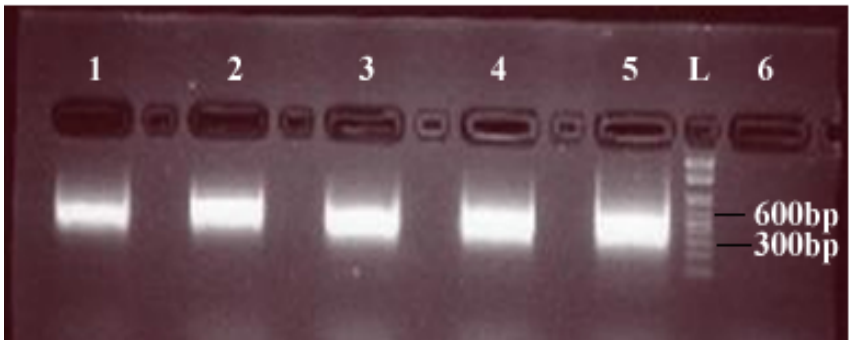


Figure 3.1: Representative PCR products of round A/B amplification
Lane 1 and 2 are from BV negative samples while lanes 3 and 4 are from BV positive samples. Lane 5 is a positive control of HeLa cells cDNA, Lane L is 1Kb ladder (ranging from 100bp to 1000bp). Lane 6 is negative control of water.

3.1.2 Cloning and sequencing of amplicons

Cloning of the gel purified products of Round B PCR was successful as evidenced by presence of many white (vector + insert) and few green (vector only) colonies on LB agar plates containing X-gal (**Figure 3.2**).

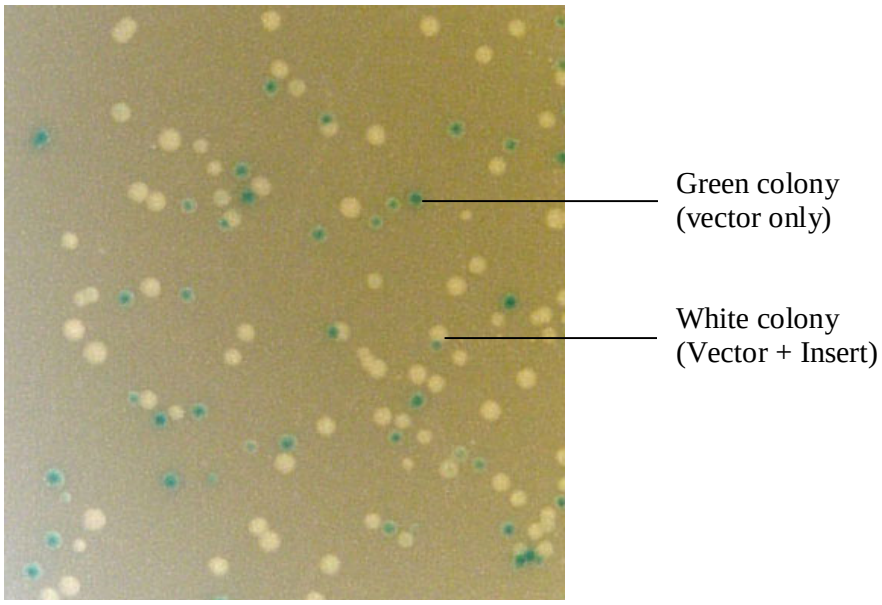
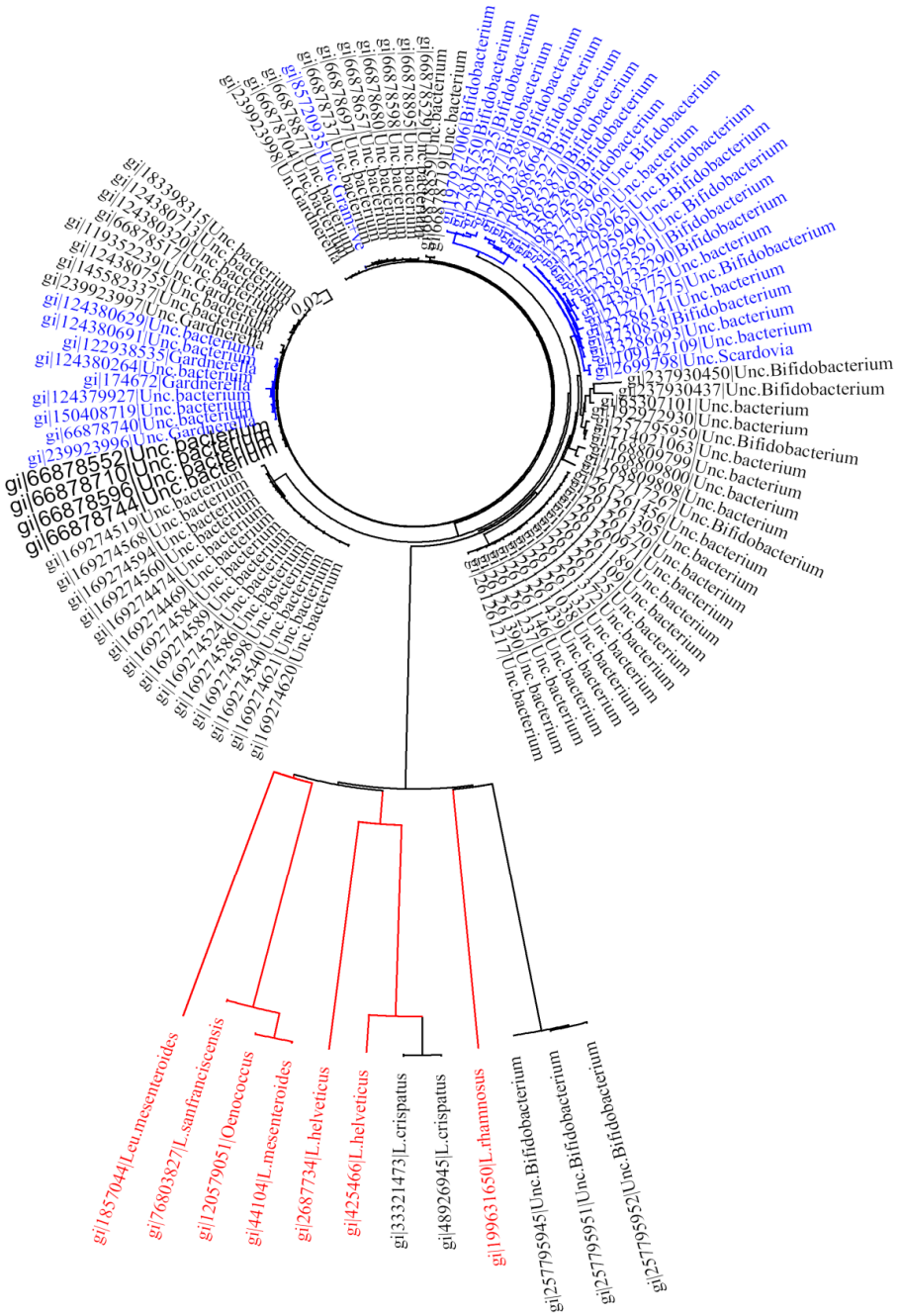


Figure 3.2: Selection of clones with inserts on Xgal
Presence of foreign DNA (insert) within the *lacZα* gene, disrupts the production of functional β-galactosidase thus inhibiting production of β-galactosidase enzyme. The enzyme metabolizes galactose to form an insoluble product 5-bromo-4 chloroindole which is bright blue thus indicating absence of insert.

From each of the twenty six samples 95 white colonies were picked for a 96 well PCR reaction with primers M13F and M13R. The PCR products within a single sample were of varied amplicon sizes ranging from 300bp to 600pb (**Figure 3.3**).

Figure 3.3: Representative PCR products of whole cell amplification from a single sample
Lanes 1 to 24 are representative PCR products from whole colony PCR reactions of a single sample. The products from other both BV positive and negative samples had a similar variation with products ranging between 300bp and 600bp. Lane L is 1Kb DNA ladder ranging from 100bp to 1000bp

The 96 sequences obtained from each of the 26 samples were each analysed for vector contamination using the on-line VecScreen software available on (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html). The quality of the sequences was determined using the sequence explorer light® software. Vector and quality clipped sequences were pooled and assembled using the Contig Assembly Program (Cap3) (http://mobyle.pasteur.fr/cgi-bin/portal.py) software to provide contigs for comparative purposes. The contigs were aligned to other nucleotide bases using the basic local alignment search tool (blastn) that is available on (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome). Sequences aligning to *Homo sapiens* and human immunodeficiency virus were filtered and excluded from further analysis. Majority of the unfiltered sequences aligned to uncultured bacterium sequences and only sequences from BV negative samples aligned to *odc* and *potE* genes of *Oenococcus oeni* which were distant apart from other oganisms. The relatedness of the sequences obtained was as shown in the phylogenetic tree shown below (**Figure 3.4**). *Bifidobacteria* were significantly associated with BV positive samples p=0.0472 (Fisher's exact test). Seventy five percent (9/12) of BV positive samples and 28% (4/14) of BV negative samples had *Bifidobacteria* detected in them. Majority of the bacteria detected were unculturable (**Figure 3.4**).



Key: Black- present in BV positive and negative, **Red**- present in BV negative only, **Blue**-present in BV positive only.

Figure 3.4: Phylogenetic tree of organisms identified from BV positive and BV negative samples

3.1.3 Hybridization on microarrays

No hybridization signals were visualized on the ViroChip slides loaded with the test samples (only Cy3 [red] signal was observed) (**Figure 3.5** - A) while in the control sample hybridization was visible (both Cy3 [red] and Cy5 [green] signals were observed) (**Figure 3.5** - B). Intensities obtained from the scanner genepix software were too low (value <10) for further analysis with cluster software. Control samples had intensity values greater than 1,000.

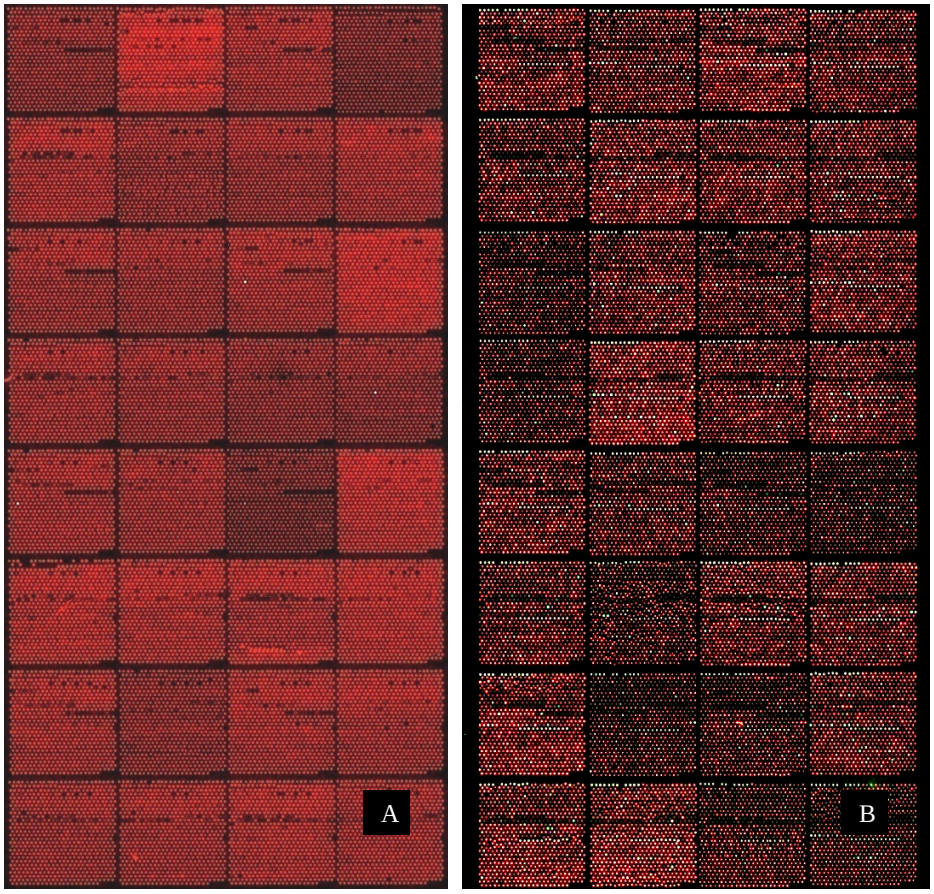


Figure 3.5: Images of ViroChip arrays captured on a genepix scanner
Red spots represents probe 70 signals and green spots represents sample signal. Hence, slide A represents a sample with no positive hit for any virus on the slide since it has no sample hybridized to any spot (green) except the control probe 70 (red). Slide B represents a sample containing viruses represented in the slide since there are spots with positive hits (green).

The control samples had intensity values above 500. The results from micro-array were valid since copies of the same samples were used for shot gun sequencing and none of the organism detectable by micro-arrays was identified by sequencing.

3.1.4 Vaginal *Lactobacilus* bacteriophages from vaginal swabs

Phage A2 was detected in two samples that were negative for BV (**Figure 3.6**). None of the other phage types A1, B1 and B2 were detected.

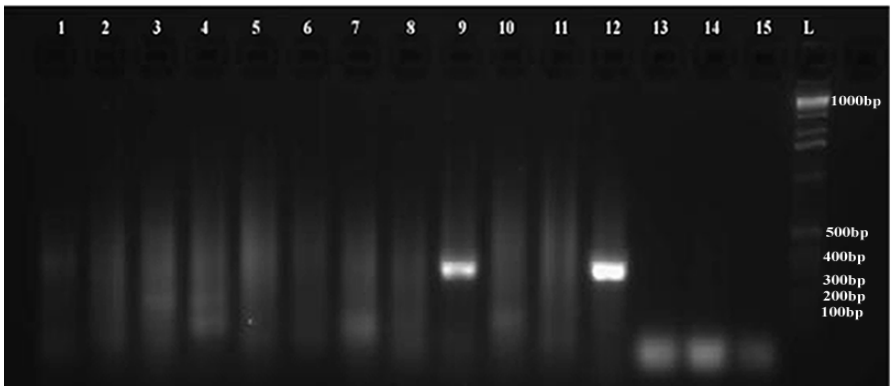


Figure 3.6: PCR products of Phage A2 from Vaginal swabs
Lanes 1 to 14 are BV negative samples, Lane 15 is negative control (water) and Lane L is 1Kb ladder (100bp to 1000bp). Lane 9 and 12 are positive for Phage type A2

3.2 Retrospective study in Kenya

3.2.1 Isolation of DNA and detection of BV associated organisms from cervicovaginal lavage

In the Kenyan study the DNA isolated from an initial 20 samples were all negative for *Mobiluncus*, *Bacteroides* and *Gardnerella* while the DNA from the positive control was positive for *Gardnerella* only. Using the same protocol, DNA was isolated from five fresh vaginal swabs obtained from a different on going study that had been confirmed to be BV positive and two that had been confirmed to be BV negative by microscopy. The DNA isolated from the seven samples was negative for *Mobiluncus*, *Bacteroides* and *Gardnerella*. To quality control the DNA extraction method a rapid method of DNA preparation using InstaGene matrix was tested in accordance with the manufacturer's instructions. Using the InstaGene matrix, the five samples that were positive for BV by microscopy had one or more of the three organisms *Mobiluncus, Bacteroides* and *Gardneralla* detected. The two BV negative samples had *Gardnerella* detected in one sample. The data from the seven samples was not shown or used for analysis in this study since they were not part of the samples with ethical clearance for the present study. DNA isolated from the spiked positive control sample using InstaGene matrix had all the three bacteria species detected. The protocol using InstaGene matrix was henceforth adopted and used for all the preceeding CVL samples that were analyzed. DNA was isolated from 490 CVL samples using the InstaGene matrix protocol. These samples had been obtained from follow up patients who had made at least four progressive visits to the clinic at intervals of approximately six months. Samples appearing bloody were not used.

Samples meeting the expected requirements from patients who attended at least the first four visits were 100 and they were from 100 different patients **Table 3.1**. Samples reduced in subsequent visits as the same 100 patients began to have inconsistent visits. The 100 patients whose samples were used were married 97% (97/100) and widowed 3% (3/100) at the time of recruitement into the study. They all reported to have never/less than half the time used a condom in the last one month and they were all below the age of 30 years.

Table 3.1: Distribution and number of CVL samples analysed

Visit Count	**No of samples**	**% of total**
First	100	20.4
Second	100	20.4
Third	100	20.4
Fourth	100	20.4
Fifth	59	12.1
Sixth	31	6.3
Total	**490**	**100**

Amplicon products from the multiplex PCR of different band sizes were observed which coincided with one or more of the expected band sizes for *Mobiluncus* (1015bp), *Bacteroides* (842bp) and *Gardneralla* (570bp) **Figure 3.7**. There were samples that had a single band or two bands coinciding with the expected size for any one of the three bacteria **Figure 3.7**. The different combinations of bands observed were run on a single gel and were as shown in **Figure 3.7** below:

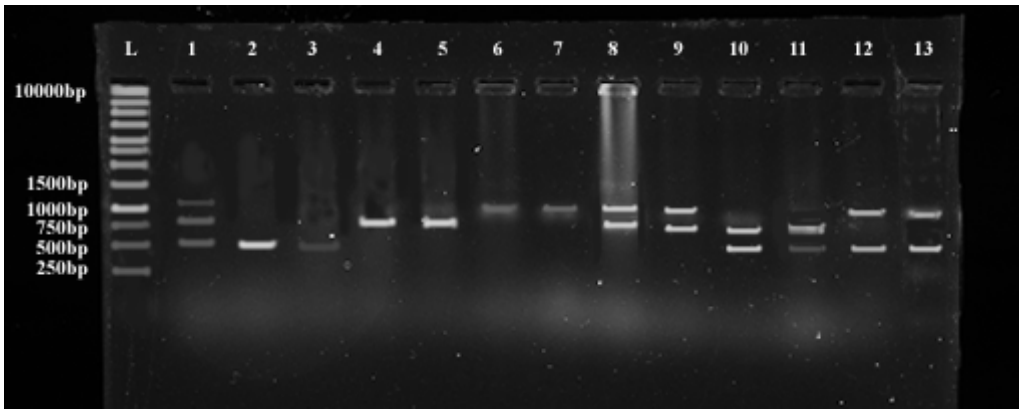


Figure 3.7: Representative multiplex PCR detection of bacterial vaginosis associated organisms

Lane L - ladder (10Kb ladder), Lane 1- positive control, Lane 2,3- *Gardnerella*, Lane 4,5 - *Bacteroides*, Lane 6,7 - *Mobiluncus*, Lane 8,9 - *Mobiluncus* and *Bacteroides*, Lane 10,11- *Bacteroides* and *Gardnerella*, Lane 12,13 - *Mobiluncus* and *Gardnerella*

Presence of *Gardnerella vaginalis* was significantly higher ($p<0.05$; by Kruskal-Wallis Nonparametric ANOVA Test) among the three organisms associated with presence of BV. Though *Gardnerella* was at a higher prevalence on post analysis using Dunn's Multiple Comparisons Test, presence of *Gardnerella* was only extremely significantly higher than *Mobilincus* ($p<0.05$) but not significantly different from *Bacteroides* ($p>0.05$). There was also no significant difference in the presence of *Mobilincus* versus *Bacteroides* ($p>0.05$) though higher percentages of *Bacteroides* were present in all the visits. The percentage distribution of the three organisms was as shown in **Figure 3.8** below, with *Gardenerela* having the highest presence.

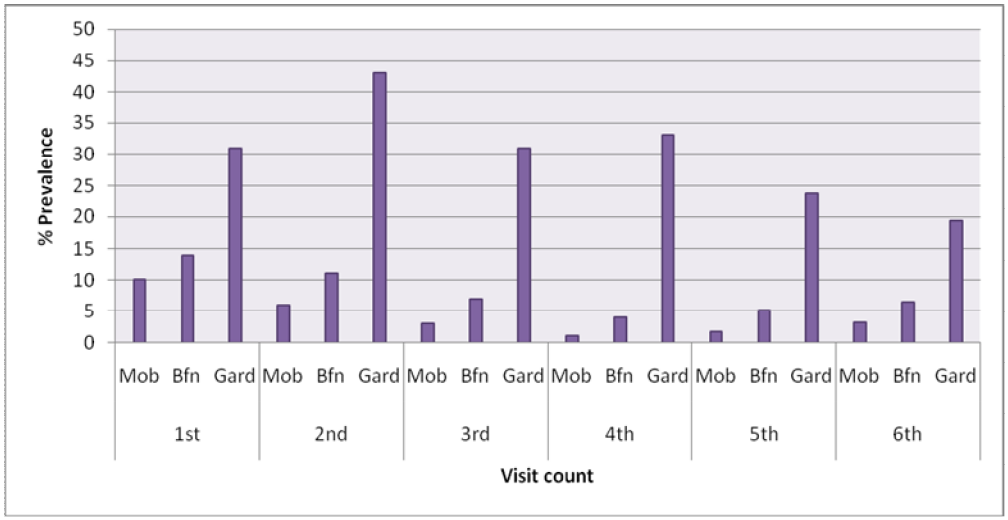


Figure 3.8: Variations in detection of *Mobiluncus*, *Bacteroides* and *Gardnerella* populations by visit.

Assuming that the presence of two or more of any of the three organisms *Mobiluncus*, *Bacteroides* and *Gardneralla* is indicative of BV presence, then there was a general decline in BV prevalence with increase in visits counts while CD4 counts had an increasing trend. The prevalence of BV ranged from 25.8% (6^{th} visit) to 51% (2^{nd} visit) (**Figure 3.9**).

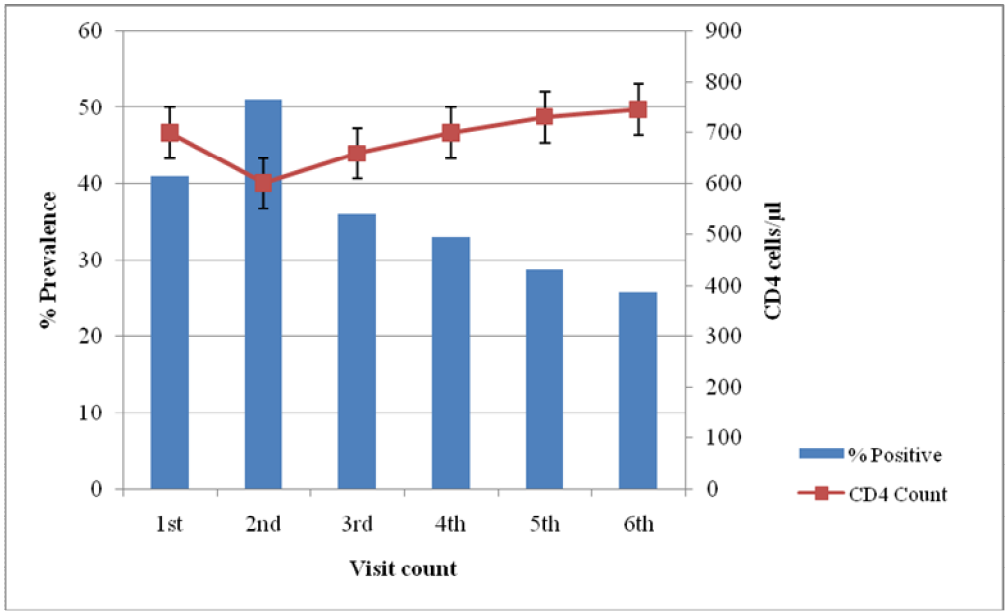


Figure 3.9: Prevalence of bacterial vaginosis and CD4 cells count by visit

The pair wise occurrence of the three organisms *Mobiluncus*, *Bacteroides* and *Gardnerella* was significantly higher in *Gardnerella* and *Bacteroides* pair ($p<0.05$; ANOVA). On post ANOVA analysis using Tukey-Kramer Multiple Comparisons Test the difference in occurence of *Gardneralla* and *Bacteroides* versus *Mobiluncus* and *Bacteroides* was significant ($p<0.05$) (**Figure 3.10**). The difference between *Gardnerella* and *Mobiluncus* versus *Gardnerella* and *Bacteroides* as well as the difference between *Gardnerella* and *Mobiluncus* versus *Mobiluncus* and *Bacteroides* was not significant ($p>0.05$) (**Figure 3.10**). Presence of both *Mobiluncus* and *Bacteroides* simultaneously was observed only from samples obtained during the first visit (**Figure 3.10**).

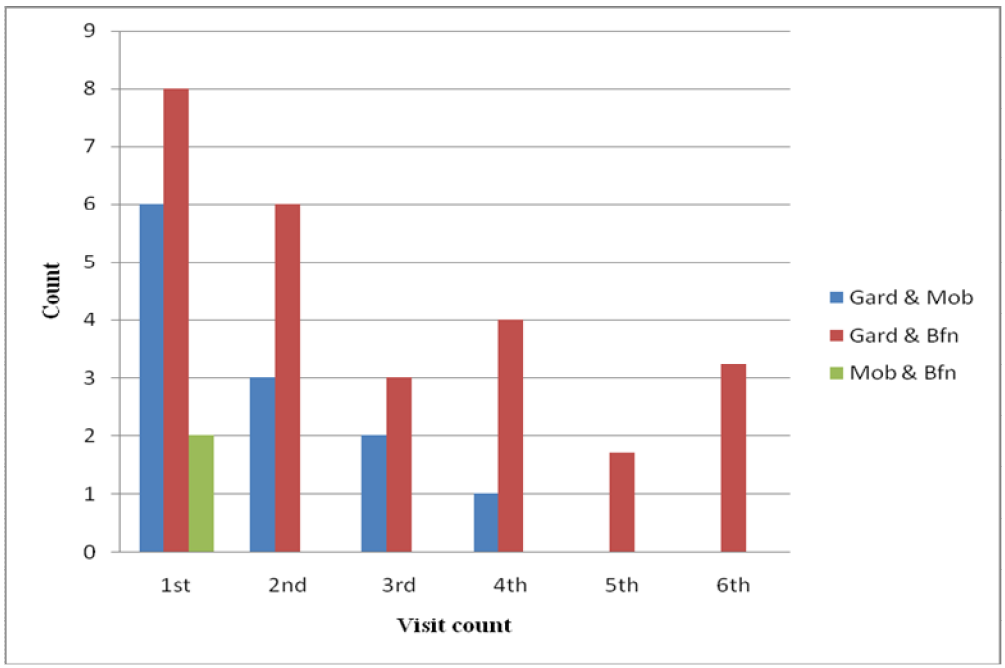


Figure 3.10: Paired occurrence of bacterial vaginosis related organisms in patients during visits

3.2.2 Detection of *Bifidobacteria* by PCR

The bacteria Bifidobacteria was detected in all categories of samples; those with two or more or without any of the detectable bacteria associated with BV. The detection of Bifidobacteria was on the basis of the presence of a 520bp amplicon (**Figure 3.11**).

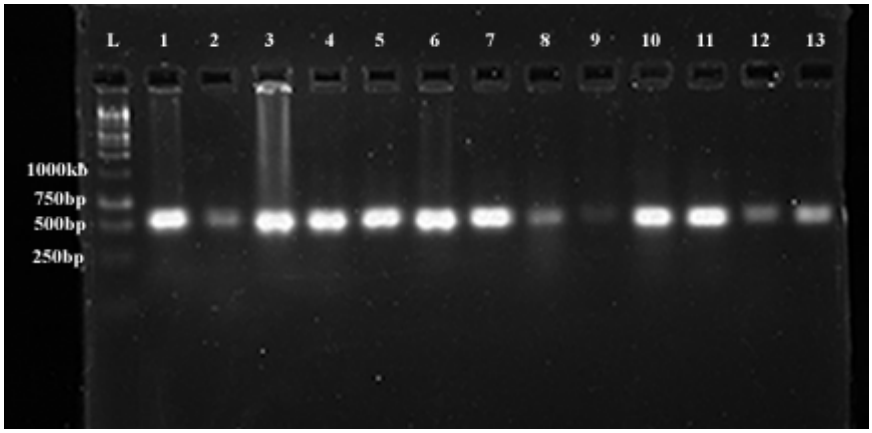


Figure 3.11: PCR detection of *Bifidobacterium* species

Screening for the presence of *Bifidobacterium* was done on 250 DNA isolates obtained from 100 samples that were negative for *Mobiluncus*, *Bacteroides* and *Gardnerella* and another 150 from samples that had two or more of the BV related organisms detected. Overall the organism was detected in 23% (58/250) of the samples tested; samples with BV associated bacteria having 16% (39/250) while those without had 8% (19/250) though, statistically the difference is not significant ($p > 0.05$; Chi-square) between the two groups (**Table 3.2**).

Table 3.2: Association of *Bifidobacteria* with bacterial vaginosis related organism

		Bifidobacteria		
		Detected	**Not Detected**	**Total**
BV Associated Bacteria	**Detected**	39 (16%)	111 (44%)	150 (60%)
	Not detected	19 (8%)	81 (32%)	100 (40%)
	Total	58 (23%)	192 (77%)	250 (100%)

Chi-square statistic = 1.281 (1 degree of freedom) p = 0.2578 (p>0.05)

3.2.3 Detection of *Oenococcus oeni* by PCR

The same DNA extracts used for detection of *Bifidobacteria* were used. Several samples gave multiple bands with the primers LeuOF/LeuOR including one band of approximately the expected size 1174 bp. Using the samples with bands a touchdown and gradient PCR were done. No optimal condition was obtained and the band of approximately 1174 bp was sequenced. The sequence obtained had

homology to *Lactobacillus helveticus* (Transposase) and *Oenococcus oeni* (*odc* and *potE* gene). The primers were re-analyzed using primer blast software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). They were found to have likelihood of amplifying *Lactobacillus helveticus* DPC 4571, complete genome (1162bp), *Oenococcus oeni* odc gene and potE gene (1177bp), *L.mesenteroides* IS1165 DNA sequence (1174bp), *Bordetella avium* 197N complete genome (1125bp), *Acinetobacter* sp. ADP1 complete genome (757bp and 117bp) among other unrelated organisms. With the exception of *Acinetobacter* sp product size the rest of the product sizes are so close on size hence, not easily resolvable on agarose gel. To confirm absence or presence of *Oenococcus oeni* new PCR-specific primer sets previously used in a nested PCR were obtained (Zapparoli *et al*. 1998). The PCR-specific primers were On1 (5'-TAATGTGGTTCTTGAGGAGAAAAT-3') and On2 (5'-ATCATCGTCAAACAAGAGGCCTT-3'); the primers On3 (5'-AATATTCAATACGAATCACG-3') and On4 (5'- GATTCCAGTTCCTTGAATA-3'). No PCR products were obtained using the nested PCR approach with primers On1/On2 and On3/On4.

3.2.4 Detection of vaginal *Lactobacilus* bacteriophages from cervicovaginal lavage

All the four phage types A1, A2, B1 and B2 were detected with type A2 having the highest percentage detection rate of 30% (30/100). Phage type B2 was detected at significantly higher rates in BV positive samples than in BV negative samples ($p<0.05$; Fisher's exact test). The samples tested for the phage presence comprised

of 50 with two or more BV related organisms; considered BV positive and 50 without any of the BV related organisms detected. The amplification products of the four types of phages were observed in varying prevalences (**Table 3.3**).

Table 3.3: Types of *Lactobacillus* bacteriophages detected

Phage type	**BV +Ve (n=50)[a]**	**BV -Ve (n=50)[b]**	**Total**	**%**	**p[c]**
A1	5	10[b]	15	15	>0.05
A2	18[a]	12[b]	30	30	>0.05
B1	3	8	11	11	>0.05
B2	20[a]	7[b]	27	27	**<0.05**

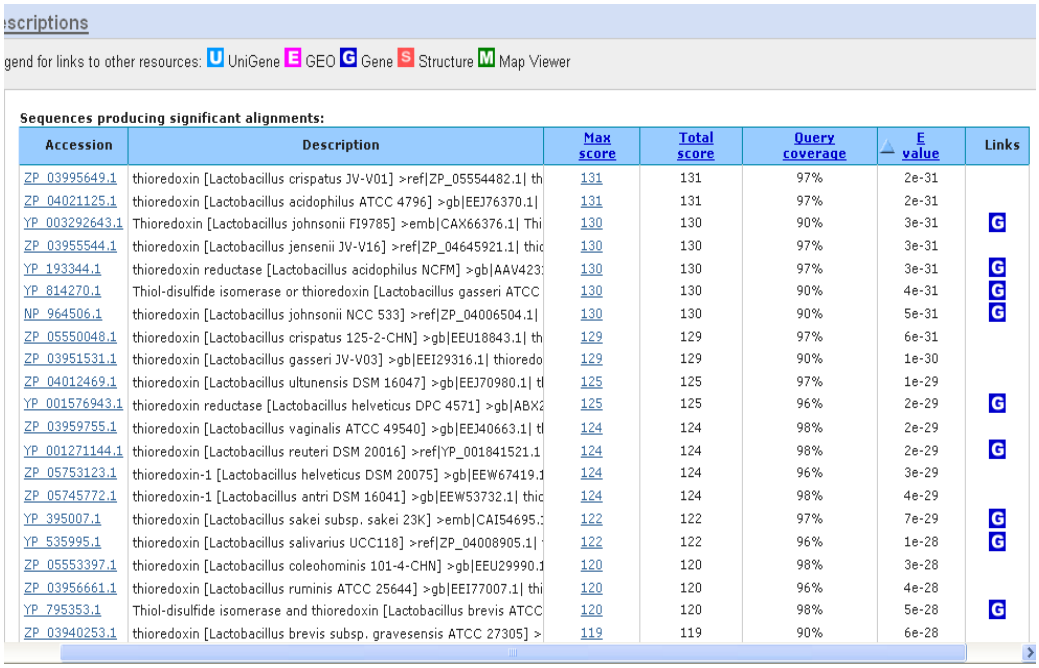
[a] Six BV positive samples had phage A2 and B2 co-detected
[b] Three BV negative samples had phage A1,A2 and B2 co-detected
[c] Fisher's exact test at 95% CL **(p<0.05 - significant)**

CHAPTER FOUR

4.0 Discussion

Metagenomic approach of shot gun sequencing has been used in identification of many novel organisms. The method allows one to sample the genomes of microbes without culturing them. It can be used both for typing and counting taxa and for making predictions of their biological functions (Eisen, 2007). In the California pilot study a metagenomic approach was therefore used resulting in many unculturable organisms being identified. Over approximately 90% of the organisms identified were unculturable bacteria (**Figure 3.4**) an indication that only less than 10% of bacteria in the vaginal flora have been studied by culture methods. A key result was the detection of *Oenococcus oeni* that has not been previously reported from the genital tract of women. The bacteria *Oenococcus oeni* (formerly called *Leuconostoc oenos*) is a lactic acid bacterium (LAB) that occurs naturally in wine, fruit mashes and related habitats (Mills *et al*., 2005). *O. oeni* is employed in wineries to carry out the malolactic conversion, an important secondary fermentation in the production of wine. *O. oeni*, a facultative anaerobe, is one of the most acid- and alcohol tolerant LAB (http://genome.jgi-psf.org/oenoe/oenoe.home.html). *Oenococcus oeni* is a Gram positive rod and is scientifically classified as follows; Kingdom: bacteria, Division: firmicutes, Class: bacilli, Order: *Lactobacillales*, Family: *Leuconostocaceae* and Genus: *Oenococcus* (Dicks *et al*., 1995)**.** *Lactobacillus* species are the most predominant Gram positive LAB in healthy vagina and their scientific classification is similar to that of *Oenococcus* with exception of belonging to the Family; Lactobacillaceae and Genus; *Lactobacillus* (Hunt and Rettger, 1930; Vásquez *et al.*, 2002). By Gram stain the two organisms might be difficult to

distinguish since they are both Gram positive rods. Hence, there is likelihood that by microscopy some *Oenococcus* may have been previously identified as *Lactobacillus*. Resistance to hydrogen peroxide by lactobacilli which gives it advantage over other bacteria that may colonize the vagina has been linked to the expression of heterologous manganese superoxide dismutase gene (Bruno-Bárcena *et al*., 2004). *Oenococcus oeni* has been shown to have *trxA* gene that is expressed on induction by hydrogen peroxide (Jobin *et al.*, 1999). The two organisms are therefore resistant to hydrogen peroxide which is an important characteristic for protection of the vagina against other microorganisms. *Oenococcus oeni* may therefore be providing an important protective role in protection against bacterial vaginosis but it has remained unrecognized. A detailed study on the organism was done using bioinformatics tools. Bioinformatics analysis of *trxA* gene in *Oenococcus oeni* by BLAST search of the gene sequence against *Lactobacillus* taxid indicates its close similarity to thiodoxine gene in *Lactobacillus* species (**Figure 4.1**).



escriptions

gend for links to other resources: U UniGene E GEO G Gene S Structure M Map Viewer

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Links
ZP_03995649.1	thioredoxin [Lactobacillus crispatus JV-V01] >ref|ZP_05554482.1| th	131	131	97%	2e-31	
ZP_04021125.1	thioredoxin [Lactobacillus acidophilus ATCC 4796] >gb|EEJ76370.1|	131	131	97%	2e-31	
YP_003292643.1	Thioredoxin [Lactobacillus johnsonii FI9785] >emb|CAX66376.1| Thi	130	130	90%	3e-31	G
ZP_03955544.1	thioredoxin [Lactobacillus jensenii JV-V16] >ref|ZP_04645921.1| thio	130	130	97%	3e-31	
YP_193344.1	thioredoxin reductase [Lactobacillus acidophilus NCFM] >gb|AAV423	130	130	97%	3e-31	G
YP_814270.1	Thiol-disulfide isomerase or thioredoxin [Lactobacillus gasseri ATCC	130	130	90%	4e-31	G
NP_964506.1	thioredoxin [Lactobacillus johnsonii NCC 533] >ref|ZP_04006504.1|	130	130	90%	5e-31	G
ZP_05550048.1	thioredoxin [Lactobacillus crispatus 125-2-CHN] >gb|EEU18843.1| th	129	129	97%	6e-31	
ZP_03951531.1	thioredoxin [Lactobacillus gasseri JV-V03] >gb|EEI29316.1| thioredo	129	129	90%	1e-30	
ZP_04012469.1	thioredoxin [Lactobacillus ultunensis DSM 16047] >gb|EEJ70980.1| th	125	125	97%	1e-29	
YP_001576943.1	thioredoxin reductase [Lactobacillus helveticus DPC 4571] >gb|ABX2	125	125	96%	2e-29	G
ZP_03959755.1	thioredoxin [Lactobacillus vaginalis ATCC 49540] >gb|EEJ40663.1| th	124	124	98%	2e-29	
YP_001271144.1	thioredoxin [Lactobacillus reuteri DSM 20016] >ref|YP_001841521.1	124	124	98%	2e-29	G
ZP_05753123.1	thioredoxin-1 [Lactobacillus helveticus DSM 20075] >gb|EEW67419.1	124	124	96%	3e-29	
ZP_05745772.1	thioredoxin-1 [Lactobacillus antri DSM 16041] >gb|EEW53732.1| thio	124	124	98%	4e-29	
YP_395007.1	thioredoxin [Lactobacillus sakei subsp. sakei 23K] >emb|CAI54695.1	122	122	97%	7e-29	G
YP_535995.1	thioredoxin [Lactobacillus salivarius UCC118] >ref|ZP_04008905.1|	122	122	96%	1e-28	G
ZP_05553397.1	thioredoxin [Lactobacillus coleohominis 101-4-CHN] >gb|EEU29990.1	120	120	98%	3e-28	
ZP_03956661.1	thioredoxin [Lactobacillus ruminis ATCC 25644] >gb|EEI77007.1| thi	120	120	96%	4e-28	
YP_795353.1	Thiol-disulfide isomerase and thioredoxin [Lactobacillus brevis ATCC	120	120	98%	5e-28	G
ZP_03940253.1	thioredoxin [Lactobacillus brevis subsp. gravesensis ATCC 27305] >	119	119	90%	6e-28	

Figure 4.1: BLAST results of *Oenococcus oeni trxA* gene against *Lactobacillus* taxid

In contrast the heterologous manganese superoxide dismutase gene associated with production of hydrogen peroxide in *Lactobacillus* is completely absent in *Oenococcus* species. BLAST search of the manganese superoxide dismutase gene sequence against *Oenococcus* taxid gives all E. value scores above 0.5 indicating no significance similarities (**Figure 4.2**).

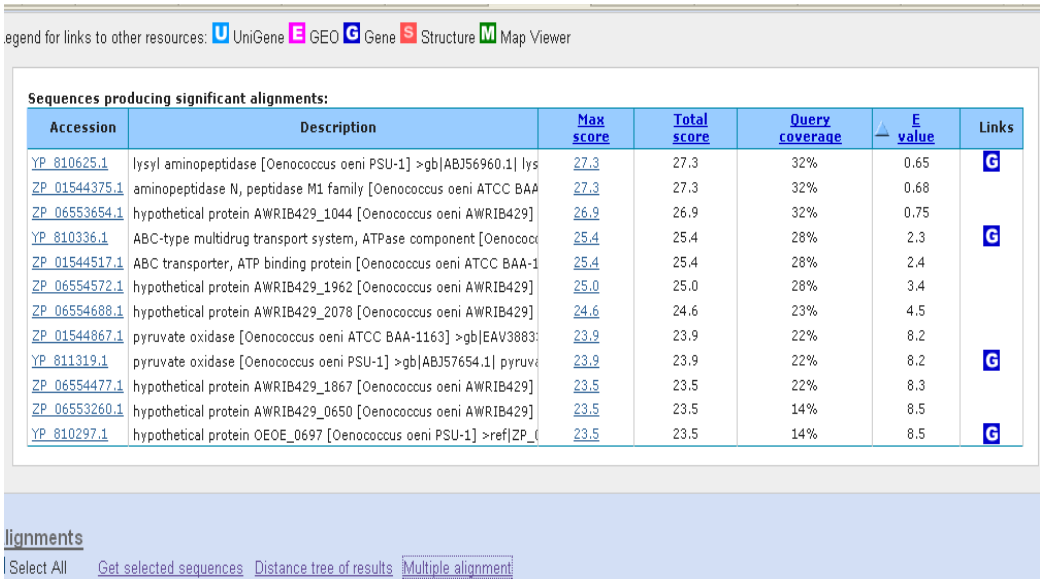


Figure 4.2: BLAST results of *Lactobacillus* manganese superoxide dismutase gene against *Oenococcus* taxid

More studies on the mechanisms in which *Lactobacillus* and *Oenococcus* resist hydrogen peroxide are important. Although, some genes may have been identified they might not be working in isolation or more so they may not be wholly responsible. In the further work done on a larger population in Kenya *O. oeni* was not detected by the use of newly designed and previously published nested primers. The newly designed primers targeted the *odc* and *potE* gene of the organism. The choice of the region was based on BLAST analysis of sequences obtained from the pilot study. The newly designed primers gave multiple products and not within the expected band size. Later on, analysis with primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast) revealed that the primers could also amplify other genes and features such as transposase, hypothetical proteins, Na+/H+ and K+/H+ antiporter, ABC transporter, ATPase component, Xaa-Pro dipeptidyl-peptidase, tRNA(Ile)-lysidine synthetase, MesJ, and Beta-galactosidase in both

Lactobacillus and *Oenococcus oeni*. The Primer BLAST results therefore explain why multiple bands were observed. The nested PCR gave no products hence confirming absence of the *Oenococcus.* Though, *O. oeni* was not detected in any of the samples there is still a need to do further investigations since this organism would be a potential probiotic since it shares many characteristics with the normal vaginal *Lactobacillus*. No information on alcohol or wine consumption by the patients is available even if permission to link the data to the samples had been sought. The organism may have chances of being acquired in the vagina after ingestion through wine consumption. To support the thought ingestion of yogurt containing *Lactobacillus acidophilus* was compared with ingestion of pasteurized yogurt as prophylaxis for recurrent BV by Shalev *et al*. (1996). Increased prevalence of the bacteria in the vagina and rectum was observed (Hilton *et al*., 1992; Shalev *et al*., 1996). With *Oenococcus oeni* being lactic acid bacteria like *Lactobacillus* are, there is likely hood that by the same mechanism it could find its way to the vagina. Oral sex has been implicated in non STI vaginal infections as well as transmission of HIV (Koelman *et al*., 2000; Gottlieb, 2000). Likewise *Oenococcus oeni* may have been introduced in the vagina through oral sex possibly after wine or coffee consumption considering that anecdotal data reveal that oral sex is predominant in California than in Kenya.

The use of different methods; shot-gun sequencing versus specific PCR may not have contributed to the differences in *Oenococcus* detection since both methods are known to have high sensitivities with detection limits of 1 pg μl^{-1} chromosomal DNA having been reported (Guo *et al*., 2004). Likewise, the sample types used

would not have affected the bacteria detection ability as it has been shown that CVLs may even be superior for detection of *Trichomonas vaginalis* over vaginal swabs (Kissinger *et al*., 2005). For diagnosis of BV, both CVLs and vaginal swabs are comparable (Kissinger *et al*., 2005). Using the report by Kissinger *et al* (2005) the reverse observation of detecting *Oenococcus* from CVLs and not from vaginal swab would have been more likely. In addition, from anecdotal data and personal observation wine consumption by women is much common in California than by women in Kenya. That may possibly explain the observation of *Oenococcus oeni* being detected in California and not in Kenya.

A study of vaginal flora of women who consume wine versus women who do not consume wine would be appropriate to determine any association and presence of the organism in the vagina.

In the pilot study, *Bifidobacterium* was associated with BV positive samples as compared to BV negative samples ($p=0.0472$). This observation was made from a small pilot study sample size. Using a larger sample size in a different population in Kenya there was no association of the organism with either presence or absence of BV. The overall prevalence of *Bifidobacteria* in the pilot study in California was 50% (13/26) while prevalence in the Kenyan study was 23%. The differences in the association of *Bifidobacteria* with BV in the two study sites may be due to the differences in the sample size. Additionally, the difference in the populations (Americans versus Africans) may also be the reason for variations in the association of the detected bacteria with BV. Similar observations by other studies have been

made in which vaginal bacterial flora from women of different races and regions vary and prevalences differ (Hillier *et al*., 1991; Hillier *et al*., 1995; Kiliç *et al*., 2001).

In a different study by Korshunov *et al.,* (1999) the composition of vaginal bifidobacteria flora in 56 clinically healthy women of reproductive age was studied. The study revealed that *Bifidobacterium bifidum, B. breve, B. adolescentis 2* and *B. longum,* were the most dominant. Nine out of 11 isolated strains were found to be capable of *in vitro* inhibition of *Staphylococcus aureus* and *Enterococcus faecalis.* In addition, strains *B. adolescentis* 2 F1, *B. breve* P2 and *B. longum* Z4 were capable of adhesion to vaginal epitheliocytes (Korshunov *et al*., 1999). This study results are thus indicative of the possibility and advantages of using bifidobacterial strains belonging to this ecological niche as probiotics for the correction of the microflora of the urogenital tract in females (Korshunov *et al*., 1999); though contradictory to the California pilot study that instead would implicate *Bifidobacteria* to play a role in BV development. It has also been reported in a different study that the contribution of *Bifidobacterium* genus to the vaginal bacterial microbiota may have been underestimated due to confusion with the more commonly detected *Gardnerella vaginalis*, an organism associated with bacterial vaginosis (Burton *et al*., 2003). To differentiate *Bifidobacterium* species and *G. vaginalis* a Denaturing Gradient Gel Electrophoresis (DGGE) method was developed by Burton *et al.,* (2003). Using DGGE it was possible to differentiate PCR products from *Bifidobacterium* species and *G. vaginalis* generated from the same primer set based upon the melting properties of their respective amplicons (Burton *et al.,* 2003). In the study using

DGGE, Bifidobacteria were infrequently detected in the vagina indicating that they are not common components of the vaginal bacterial microbiota, and may be fecal in origin, given the species detected (Burton *et al.*, 2003). This observation supports the pilot study that strongly implicates *Bifidobacterium* to be playing a role in BV. *Bifidobacteria* are sensitive to hydrogen peroxide exposure (Kheadr *et al.*, 2007) and hence their survival in the vagina may not be possible. A BLAST search of the genes associated with hydrogen peroxide resistance and production in *Lactobacillus* against *Bifidobacteria* taxid, complements the findings of Kheadr *et al*. (2007) since no significant alignments were observed (E. value scores were above 0.5) though a thioredoxin gene is present. In addition, *Bifidobacterium* has been found in high prevalence among samples tested positive for BV by Nugent method agreeing with a different study in pregnant women where the organism was present in 94% of BV positive, 58% of intermediate BV and 12% of healthy controls (Rosenstein *et al.*, 1996). In contrast, a different study reported the importance of *Bifidobacteria* species as a probiotic in correction of vaginal flora (Korshunov *et al*., 1999).

The presence of *Bifidobacteria* in the vagina with the presence of hydrogen peroxide producing *Lactobacilus* may be questionable.

In a separate study Hyman *et al.* (2005) studied twenty healthy premenopausal women aged 27–44 years at various (recorded) times of their menstrual cycle. The women had no complaints of urogenital symptoms or noticeable infection on physical examination of the urogenital tract (Hyman *et al*., 2005). Using solely a gene-based procedure, PCR amplification of the 16S ribosomal RNA gene coupled

with very deep sequencing of the amplified products was done on the vaginal samples from the 20 women. In four subjects, *Lactobacillus* was the only bacterium detected. Eight subjects presented complex mixtures of *Lactobacillus* and other microbes. The remaining eight subjects had no *Lactobacillus*. Instead, *Bifidobacterium*, *Gardnerella*, *Prevotella*, *Pseudomonas*, or *Streptococcus* predominated (Hyman *et al*., 2005). If the eight subjects who had no *Lactobacillus* were to be screened by Gram stain and scored by Nugen *et al*. (1991) criteria they would be considered BV positive or intermediate. Similarly, using the PCR method of Obata-Yasuoka *et al.*, (2002) they would also be considered BV positive. By clinical observation as described by Hyman *et al*. (2005) the women had no symptoms of any vaginal infections and they were not on any contraceptives. This adds to the complexity of what really defines BV. One question that begs for an answer is; is *Lactobacillus* realy significant for existence of a healthy vagina with no signs of infections? Two answers may exist; yes and no. The answer would be no in view of studies recommending use of *Bifidobacteria* as probiotics to correct vaginal flora (Korshunov *et al*., 1999) and the absence of any vaginal infections in vaginal flora with no *Lactobacillus* but predominated with *Bifidobacteria* (Hyman *et al*., 2005). The answer would be yes in view of studies that *Bifidobacteria* are sensitive to hydrogen peroxide (Kheadr *et al*., 2007) while *Lactobacillus* are resistant and producers of hydrogen peroxide which is inhibitory to other micro organisms (Bruno-Bárcena *et al*., 2004). Hence, *Lactobacillus* cannot co-exist with *Bifidobacteria* and it is of more importance in the vagina. Based on the current study findings the role of Bifidobacteria in the vagina cannot be identified. Full genome sequencing of the *Bifidobacteria* strains found in symptomatic and asymptomatic

vagina should be done. This would provide an insight on whether there exist unique beneficial and non beneficial strains of Bifidobacteria. With the presence of some *Bifidobacteria* species clustering with *Lactobacillus* species and some clustering with *Gardenerella* species there is strong indication of completely distinct species existing.

The virochip was not able to pick any organism. It is based on viruses nucleotide sequences (Chiu *et al.*, 2008) and considering that by shot gun sequence none of the viruses detectable by the virochip was identified is an indicator of its reliability.

In the pilot study bacteriophages were detected in two samples that were BV negative. The phage prevalence was 2/26 (8%) and correcting for BV negative where *Lactobacillus* are present in high numbers 2/14 (14%). Though, no statistical tests could be done due to the low sample size, a quick glance at the results may be indicative that with a large sample size, the detection of free phage by PCR would be feasible. These was confirmed in a larger sample size in Kenya where the four bacteriophage types (A1, A2, B1 and B2) were detected by PCR on DNA extracted from filtrates of cervical vaginal lavage washes. The presence of phages A1, A2 and B1 could not be associated with BV status ($p>0.05$). The phage B2 was significantly associated with BV presence ($p<0.05$). The association of phage B2 with BV may have occurred by chance since by pooling all the phage types into one and retesting the association the significant association with BV is lost ($p>0.05$).

The phages targeted for detection by PCR have previously been described and were detected after induction of *Lactobacillus* using mitomycin C (Kiliç *et al*., 2001). In the present studies no cultures were done and hence, mitomycin C induction was also not done. One objective of the present studies was to detect free phage that may be present in the vagina. Detection of phages in vaginal samples in both California and Kenyan studies without a prior step of culture and induction with Mitomycin C as was done by Kilic *et al*. (2001), is a significant observation. It might be that on thawing of the samples from -80^{o}C to 4^{o}C before filtration may have induced the release of the phages. It may also be that they were frozen as free phages from the time of sample collection. The present study design only confirms presence and the ability of phage detection.

The presence of all the four phage types among the Kenyan women infected with HIV is similar to that reported among women from the United States of America (HIV status not mentioned) (Kiliç *et al*., 2001). Contrary to the observations made in the Kenyan study and the one reported on women in the United States, women from Turkey had only A1, B1 and B2 types of the phages detected (Kiliç *et al*., 2001). The distribution of the phage types does therefore vary by region and is also likely to vary by HIV status. Lysogenic *Lactobacillus* have been shown to spontaneously release phages which have a broad host range and can be lytic against other vaginal lactobacilli regardless of their geographical origin (Kiliç *et al*., 2001). Lactobacilli from yogurt have also been shown to release phages that are inhibitory to vaginal lactobacilli (Tao *et al.,* 1997). The resistance of the lactobacilli to the particular phage that is present in the vagina may not determine whether BV will develop or

not and hence the mere identification of the phage type present may not firmly establish the association with BV.

Isolation of DNA for use in classification of Kenyan samples into BV positive and negative as well as the determination of BV prevalence was not successful using the method described by Obata-Yasuoka *et al*. (2002) as simple and cost effective. The method involves boiling of normal saline suspensions of vaginal swabs to obtain a template for PCR (Obata-Yasuoka *et al*., 2002). It is not clear to explain why the method was ineffective in the present study. It may be that the method is effective only in freshly collected samples and not effective in frozen samples. Further studies should be carried out to resolve this problem. InstaGene matrix which uses a similar procedure to that described by Obata-Yasuoka *et al.* (2002) with exception of using the matrix instead of normal saline provides a cheap and quick alternative.

Fewer samples were used for fifth and sixth visits which was partially due to patient fall out. In addition, some patients commenced midway the project and hence they could not get to fifth and sixth visits before the end of the project. The patients had a significant improvement in their CD4 counts as time progressed (**Figure 3.9**) which was mainly attributed to dietary education and antiretroviral drug therapy provided at no cost (Dr. Mwachari, personal communication, April, 2009). The feel of recovery to good health may also have contributed to some patients not complying. The improvement in CD4 counts supports the percentage decrease in BV prevalence with time progression. The decline in presence of BV associated organisms assumed to be BV occurrence in the current study is therefore likely to be due to improved health

and boost of immunity. In a different study it was observed that in a HIV high risk population, clinically diagnosed BV is independently correlated with HIV prevalence and therefore, BV may either increase female susceptibility to heterosexual HIV transmission, or be a marker for altered lower genital tract immunity (Cohen *et al.*, 1995). In the same study when the bacterial vaginosis diagnosis was based on Gram stain (score 7-10), the association with HIV seropositivity disappeared, but having abnormal vaginal flora (gram stain score 4-10) was related to HIV status (Cohen *et al.*, 1995). A standardized method for diagnosis of BV is therefore important for any accurate correlation of BV to HIV.

Gardnerella was the most prevalent of the three organisms associated with BV in the present studies. Its high presence confirms previous observations by other investigators but does not provide any answers to the role it has in BV development. Its role in BV development has been in question for over half a century. The organism was first reported as a cause of bacterial vaginitides (now bacteria vaginosis) in 1955 (Gardner and Dukes, 1955). These investigators believed that *G. vaginalis* was the sole cause of BV and set out to fulfill Koch's postulates for disease causation in a series of clinical experiments. Pure cultures of *G. vaginalis* were inoculated into the vaginas of 13 healthy women, which resulted in the development of BV in 1 of the 13, with a corresponding rate of disease production of 7.7%. Based on these data, the investigators concluded that Koch's postulates were fulfilled, though the 92% failure rate calls this conclusion into serious question (Srinivasan and Fredricks, 2008). The investigators performed additional experiments wherein whole vaginal fluid obtained from subjects with BV was used to inoculate the

vaginas of 15 women without BV. Eleven of these 15 subjects developed BV. The authors felt that these data further supported the causal role of *G. vaginalis* in BV because this bacterium was cultured from most of the induced cases (Srinivasan and Fredricks, 2008). Srinivasan and Fredricks (2008) therefore interpreted these findings to conclude that whole vaginal fluid is a much more successful inoculum for the transmission of BV than is a pure culture of *G. vaginalis.* Other studies have shown that prevalence of *G. vaginalis* is significantly higher in presence of BV (96%) but it is also present in absence of BV (70%) (Fredricks *et al*., 2007). The specificity of using *G. vaginalis* as an indicator of BV is therefore poor but when combined with detection of one of the Clostridiales bacteria (BVAB2) or *Megasphaera* type 1 the best sensitivity and specificity for PCR diagnosis of BV was obtained, regardless of the gold standard diagnostic criteria employed (sensitivity 99% and specificity 89%) (Fredricks *et al.*, 2005; Fredricks *et al.*, 2007).

In the Kenyan study detection of *G. vaginalis* was combined with detection of either or both *Mobiluncus* and *Bacteroides* as indicators of BV hence, the prevalences obtained for BV in the Kenyan study are likely to differ if other methods were to be used. The PCR method described by Obata-Yasuoka *et al*. (2002) was preferred in the present study since frozen samples were used as opposed to Gram stain method which though the gold standard, requires samples to be processed soon after collection. The PCR based method does not require specialized training and is easily reproducible unlike the Gram stain method which though highly reproducible using Nugent *et al*. (1991) method, requires specialized training and extra caution during slide prepations (Joesoef *et al*., 1991). Using the Gram stain as the gold standard the

PCR based method has 78.4% diagnostic sensitivity, 95.6% specificity, 94.2% negative predictive value and 81.9% positive predictive value (Obata-Yasuoka *et al.*, 2002). The high negative predictive value implies the method is appropriate for screening; however considering that 18.1% (6/35) of the patients with positive results were overdiagnosed the method would be most efficacious for diagnostic use when used in combination with the clinical diagnostic criteria (Obata-Yasuoka *et al.*, 2002). The method was used for screening purposes and mainly for identification of the three common organisms associated with BV which are equally the same organisms (Nugent *et al.*, 1991) targets in the Gram stain method. The prevalences of BV in this study are therefore, also likely to be overstated. In addition, Obata-Yasuoka *et al.* (2002) considered a sample BV positive when one or more anaerobes were positive in the vaginal sample. In the present study samples with two or more anaerobes detected were selected for association studies with *Bifidobacteria*, *Oenococcus* and bacteriophages. Mathematically this improves the positive predictive value, hence bringing down the number of overdiagnosed samples in the association studies.

Vaginal swabs were collected and transported to the laboratory in a cool box at 4 ^{o}C for the study in California while the CVL samples were collected on site and were aliquoted and stored in -80 ^{o}C immediately for the study in Kenya. In California the samples were processed on arrival to the lab while in Kenya the samples had been frozen at -80 ^{o}C for two to three years at the time of use. Several studies have determined the effect of temperature on stability of a number of viruses with most reporting that 4 ^{o}C is suitable for short term storage (Blackwell and Barlow, 1984;

Domeika and Drulyte, 2000; John *et al*., 2001; Rosa-Fraile *et al*., 2005; Legoff *et al*., 2006). For instant Legoff *et al.* (2006) determined storage temperature effects on stability of HIV-1 RNA and HSV-2 DNA in cervicalvaginal secretions collected by vaginal washing (CVL). Their findings were that HIV RNA levels decreased slightly after 72 hours in samples stored at 4 ^{o}C and −20 ^{o}C while those stored at −80 ^{o}C remained stable. The levels of HIV-1 RNA in samples stored at 20 ^{o}C and 30 ^{o}C were significantly lower after 48 hours (P = 0.01), with respective decreases of 0.79 log and 1.39 log (Legoff *et al*., 2006). Studies on Group B streptococci (GBS), which is a fastidious bacterium, have also reported its stability when stored at 4 ^{o}C (Rosa-Fraile *et al*., 2005). From swabs kept at 4°C, GBS colonies were recovered from the same 41 samples after 24, 48, and 72 hours (100%) and redused to 39 samples after 96 h (95%). The storage temperatures used in the present study are therefore not likely to have had any effect on the detectability of the bacteria and viruses. Hence, there is a stronger leaning on population differences being the cause of the variations in the different bacteria types detected. Additionally, with evidence of diet (Hilton *et al.*, 1992), personal hygiene (Hutchinson *et al*., 2007) and personal habits such as smoking (Pavlova and Tao, 2000) affecting the vaginal bacteria flora they are all likely to be reasons for the variations observed between the two study cohorts from California and Kenya.

CHAPTER FIVE

5.0 Conclusions and Recommendations

5.1 Conclusions

The following conclusions can be made from the study:

1. Unculturable bacteria are the largest population of micro-organisms in the vagina of women with and without BV.

2. This study reports the first isolation of *Oenococcus oeni* from the vagina of women without BV. No other published reports have reported the isolation of the bacteria from the vagina.

3. This study reports direct detection of vaginal *Lactobacillus* bacteriophages by PCR without prior culture and induction with mitomycin C as in previously published reports.

4. The trend in prevalence of BV was inversely related to CD4 counts trends. As the prevalence declined with respect to visit count the CD4 counts increased.

5. *Gardnerella vaginalis* is the most prevalent of the three most common bacteria associated with BV.

6. There are *Bifidobacteria* species that are associated with presence of BV and others that are associated with absence of BV.

7. Detection of BV associated organism by PCR provides an effective screening method for BV. In addition, use of multiplex PCR is a feasible method for use in detection of BV in resource limited settings compared to shot gun sequencing especially in samples stored frozen for long periods of time.

8. Suspension of swabs in phosphate buffered saline (PBS) and transportation in a cool box at 4 ^{o}C is efficient in maintaining nucleic acids integrity.

9. Phage type B2 was significantly associated with presence of BV while the other types A1, A2 and B1 were not associated with presence or absence of BV.

10. Isolation of DNA from cervicovaginal lavage for PCR screens by the normal saline boiling method was not effective.

11. This study has confirmed that the use of RNAse and DNAse treatment is not sufficient to eliminate host DNA/rRNA since Homo sapiens sequences were detected in all samples

12. The use of the Virochip micro arrays as described in the present study does not provide an efficient platform for detection of uncharacterized microorganisms from the polymicrobial vaginal flora.

5.2 Recommendations

1. The use of anti-retroviral drugs has direct impact on improvement of CD 4 cells count which subsequently results in reduction of BV prevalence. It is therefore, recommendable that women infected with HIV comply with ARVs medication.

2. *Oenococcus oeni* needs to be investigated further by researchers as a potential probiotic for treatement of BV. The bacterium was identified in BV negative samples and it shares similar characteristics to the normal vaginal *Lactobacillus* species.

3. Previouse studies on BV have focused on culturable bacteria. The present study has shown that unculturable bacteria are the most predominant bacteria in the vagina. A shift to studies on unculturable bacteria would be the way forward to better understanding of BV. It would be vital to develop methods for drug susceptibility testing of unculturable bacteria or develop new culturing methods.

4. Studies on lactobacilli lysogenic bacteriophages have been limited to culturable lactobacilli since they require induction with mitomycin C. Lysogenic phages in non culturable vaginal lactobacilli should be studied by direct PCR.

5. Studies should be mounted to determine the correlation of wine consumption and detection of vaginal *Oenococcus oeni.*

6. The occurrence of *Bifidobacterium* species especially in the absence of BV needs to be investigated further. There exist new strains or variants of *Bifidobacterium* species that are closely related to *Lactobacillus* species while others are close *to Gadnerella* species. Further studies should be done to confirm the classification of *Bifidobacterium* species.

7. Further research is needed in order to develop effective methods for eliminating host nucleic acids with a view to increasing the sequence coverage of microorganisms.

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APPENDICES

Appendix 1: Consent to storage of samples for future research

KENYA MEDICAL RESEARCH INSTITUTE/WORLD HEALTH ORGANIZATION
CONSENT TO STORAGE OF BLOOD AND SALT WATER WASH (CVL) FOR FUTURE RESEARCH

Investigators	Title	Phone
Dr. Christina Mwachari	Co-Principal Investigator	714579 (Nairobi)
Dr. Craig Cohen	Co-Principal Investigator	714851 (Nairobi)

We would like to keep the samples of blood and salt water wash of the vagina (CVL) that are left over after the completion of the study you are participating in now, for possible future research. If you agree, these samples will be frozen and stored and possibly utilized for research in coming years when a new question may arise about HIV infection. Such questions can often be addressed and answered by analyzing stored samples. Thus, your blood and CVL samples may be helpful for this research study whether you do or do not have HIV infection.

The research that may be done with your stored blood of CVL samples probably will not help you directly, however, the research may be of benefit to other HIV infected people in the future.

Reports about research being done with your stored blood and CVL samples will not be given to you or your doctor. These reports will not be put in your health record. The choice to let you keep the left over blood and CLV for future research is up to you. No matter what you decide to do it will not affect your care. If you decide now that your blood and CVL can be kept for research, you can change your mind at any time. Just contact us at the numbers above and let us know that you do not want us to use your blood of CVL. Then the blood of CVL will no longer be used for research.

In future, people who do research on the stored samples may need to have more information about your health situation at the time the blood and CVL samples were collected. Therefore, the study co-ordinator may be asked to provide such information, although your name will not be revealed when such information is given.

Sometimes blood is used for research of genetic factors (about disease that are passed on in families) in relation to HIV infection. Even if your blood is used for this kind of research, the results will not be put in your health records.

Your blood and CVL will be used only for research and with you blood and CVL may help to advance knowledge or develop new and better products and medicines to improve health care in the future.

Please read each sentence below and think about your choice. After reading each sentence, circle "Yes" or "No", or "I agree" or "I do not agree". No matter what you decide to do, it will not affect your care. If you have any questions, please talk to your doctor or nurse or please contact the doctor listed at your study site below.

1. My blood and CVL samples will be stored indefinitely with a linkage to my research records.

   Yes No

2. My samples will be used for research to learn about, prevent, treat of cure HIV/AIDS.

   Yes No

3. I may withdraw my samples from storage at any time.

   Yes No

4. I consent to the storage and future testing of my blood and CVL samples as outlined above.

   I agree I do not agree

Please sign your name here after you circle your answer.

Your Signature: _____ Date _____

Signature of Doctor/Nurse: _____ Date _____

Witness: _____ Date _____

I certify that this is an accurate and true translation

Name: _____
(signature of recruiting doctor or nurse)
Address:
Phone:
*If you would like to with draw your samples from storage, please contact your study co-ordinator who will then contact Dr. Judith Franke at the CDC Lawrenceville Facility.
Tel: 770-339-5942

Appendix 2: Round A/B/C Random Amplification of DNA

Protocol DeRisi Lab, UC San Francisco, June 2001

This protocol was adapted from Bohlander *et al*. *Genomics* 13 (1992).
The goal of this procedure is to randomly amplify any given sample of DNA which as much representation as possible. It is not a "linear" method, but is useful to compare relative enrichment between two samples. This protocol has been used successfully to amplify genomic representations of less than 1ng of DNA. The protocol consists of three sets of enzymatic reactions. In Round A, sequenase is used to extend randomly annealed primers (Primer A) to generate templates for subsequent PCR. During Round B, the specific primer B is used to amplify the templates previously generated. Finally, Round C consists of additional PCR cycles to incorporate either amino allyl dUTP or Cy-dye-coupled nucleotide.

Caution: Wear gloves and be careful of contamination as ANY DNA can be amplified by this protocol. Use filter tips!!! *Always* run a control sample with water only (no DNA template) to make sure the reagents are not contaminated with DNA.

Materials

Round A
Sequenase (13 units/µl) US Biochemical cat# 70775
5X Sequenase Buffer
Sequenase Dilution Buffer
3 mM dNTP mix
500ug/ml BSA
0.1 M DTT
40 pmol/µl Primer A: GTT TCC CAG TCA CGA TCN NNN NNN NN

Round B
10X PCR Buffer (500 mM KCl, 100 mM Tris pH 8.3)
25 mM MgCl2
100X dNTPs (20 mM each nucleotide)
5 unit/µl Taq polymerase
100 pmol/µl Primer B: GTT TCC CAG TCA CGA TC

Round C
Same as Round B except use modified dNTP mix
100X modified dNTP mix
25 mM dATP
25 mM dCTP
25 mM dGTP
10 mM dTTP
15 mM aminoallyl-dUTP or Cy-dUTP
(The ratio of aa-dUTP to dTTP can be altered/optimized)

Protocol

1. Round A Reactions
Denature template DNA/primer annealing
As little as 10 nanograms of DNA can be effectively amplified by this protocol.

7 µL DNA
2 µL 5X Sequenase Buffer
1 µL Primer A (40 pmol/µl)
Total Volume = 10 µL

Heat 2 min at 94 °C, Rapid cool to 10 °C and hold 5 min at 10 °C
Add Reaction Mixture to sample:

1 µL	5X Sequenase Buffer
1.5 µL	3 mM dNTP
0.75 µL	0.1 M DTT
1.5 µL	500 ug/µl BSA
0.3 µL	Sequenase (13U/µl)

Total Volume = 5.05 µl

Ramp from 10 °C to 37 °C over 8 min. Hold at 37 °C for 8 min; rapid ramp to 94 °C and hold for 2 min. Rapid ramp to 10°C and hold for 5 min at 10 °C while adding 1.2 µl of diluted Sequenase (1:4 dilution). Ramp from 10 °C to 37 °C over 8 min. Hold at 37°C for 8 min.

Dilute samples with water to final Volume = 60 µl.

2. Round B PCR

Round A Template	7ul
MgCl2	4ul
10X PCR Buffer	5ul
100 X dNTP	1ul
Primer B(100pmol/µl)	1ul
Taq	1ul
Water	31ul

Round B PCR Cycles:
30 sec 94 ℃
30 sec 40 ℃
30 sec 50 ℃
2 min 72 ℃

Run 15-35 cycles, depending on the amount of starting material.

Run 5 µl on 1% agarose gel. A "smear" of DNA should be present between 500bp –1kb.

It may be necessary to remove aliquots every 2 cycles to check the amplification in order to optimize the number of cycles. It is best to use the minimal number of cycles that generates a visible shmear. Make sure there is no DNA in the negative control lane!

3. Round C
Use 10-15 µL of Round B to seed the Round C reaction:

Round B Template	5-10ul
MgCl2	4ul
10X PCR Buffer	5ul
100X aa-dNTP/cy-dNTP	1ul
Primer B(100pmol/µl)	1ul
Taq	1ul
Water	29-34 ul

Round C PCR conditions
30 sec 94 ℃
30 sec 40 ℃
30 sec 50℃
2 min 72 ℃ (even longer extension times may improve yield if directly coupling Cy dyes)

10-25 cycles can be run
If aa-dNTPs were used in Round C, the sample must be desalted (to remove Tris buffer which interferes with the coupling) prior to dye coupling. Add 400 µL water to the sample in a Microcon 30, and spin (about 8 min, 12K). Repeat once with 500 µl water.

Appendix 3: Post processing of micro array slides

Before starting:

1. Gather and clean containers you'll need for post-processing:
   4 glass chambers
   4 lids for glass chambers (metal lids also OK)
   2 500ml cylinders (plastic OK)
   1 100ml cylinder (plastic OK)
   1 stir bar
   1 500ml glass beaker
   1 metal slide rack

Clean each of above w/ 3 rinses of distilled water, 1 rinse of 95% EtOH, air dry on paper towels. (Note: For the sake of time, I always 1st clean up 1 500ml cylinder and 1 glass dish and lid and set aside while doing other cleaning. That way, when I'm finished with all the other stuff, I've got the containers ready for making the 'shampoo' solution that needs to be incubated for ~ 30min minimum at 65°C before starting the post-processing)

2. Turn on 37°C humidifier (so it's ready for hydration step when you get to it!).
3. Turn on heat block to high (so it's ready for snap-drying step when you get to it!).
4. Make up 'shampoo' solution in cleaned and dried 500ml cylinder:

Final [Reagent]	**Stock [Reagent]**	**Vol for 350ml**
3X SSC	20X	52ml
0.2% SDS	10%	7ml
H_2O	----	292ml

Transfer to clean, dry glass chamber. Cap with lid and transfer to 65C water bath.

5. Make up 100ml of 0.5X SSC solution (2.5ml 20X SSC, 97.5ml MilliQ H_2O) for hydration resevoir using cleaned, dried 100ml cylinder. Transfer to resevoir.
6. Check to make sure there is a stock of 1M NaBorate, pH 8.0 on hand. If not, make up this solution:
   61.83g Boric acid into 700ml MilliQ H_2O
   Adjust pH with NaOH
   (this requires ~ 20ml of 10N NaOH, and ~25ml 2N NaOH)
   Bring to 1L with MilliQ H_2O
   Filter sterilize

7. Prep arrays: after shampoo treatment, you will no longer be able to see the array. So, you need to mark the top and bottom edges to know where to place coverslips when using the array. Do this on the back side of the array with the diamond knife. Try to make a clean clear line that is visible from the

front side of the slide, don't saw back and forth a lot—this can interfere with the image after scanning.

8. Hydrate arrays: insert the arrays into the slide slots, array side down (facing the water) above the resevoir. Incubate until you see the spots changing (sort of like the lights coming on in a skyscraper at night…)—a more scientific way to do this is to actually look at the spots under a dissecting scope, but no one really does this. Try to time how long this takes for the first 1-2 sets, then simply be consistent about incubation time above the resevoir before snap-drying. (For our 20K spotted Viro3 array, this takes less than 30sec if the resevoir is preheated.)
9. Snap-dry arrays: after hydrating, immediately transfer slide (array side up!) to heat block for snap drying. Bend over quickly and watch—you should see the water on the array evaporate. It usually happens pretty quickly. You probably don't need to leave on for more than 3 secs. Transfer to slide box.
10. Use the stratalinker to UV crosslink the oligos to the slide. Transfer 15 slides maximum at a time, array side up, onto the Whatman paper in the stratalinker. Turn on the machine. Use the following settings: Energy=700μJ (x100). Press start. The machine should count down rather quickly from 700 to 0, then beeeeeeep. Press reset, transfer arrays to metal slide rack. Repeat until all slides have been cross-linked and transferred to slide rack.
11. Fill up 1 of the glass chambers will ~350ml of MilliQ H_2O. Place a piece of tape on the outside of the chamber labeled 'H_2O'. Cover this and place near the 65^oC water bath where the shampoo is warming.
12. Fill up the other glass chambers will ~350ml of 95% EtOH (!make sure the EtOH is crystal-clear, with no particulates!!). Cover this and place next to the H_2O chamber by the 65^oC water bath.
13. Shampoo: Grab a slide rack dipper, the metal rack full of slides, and pull out the pre-warmed shampoo chamber. Dunk the slides into the shampoo solution 20-40X, then cap with glass lid, and return to 65^oC water bath. Incubate 5min.
14. Washing: After 5min, uncap the water and EtOH chambers. Remove the shampoo chamber from the 65^oC bath, uncap and then using the slide rack dipper, transfer the slides to the water chamber, dunk 20-40X, and incubate 1min. Use the slide rack dipper to transfer the slides to the EtOH chamber, dunk 20-40X, and then spin 600rpm in the tabletop Beckman to dry.
15. Succinic anhydride prep: While spinning, grab a 15ml conical tube and aliquot 15ml of the 1M NaBorate solution and bring to the chemical room. Measure 5.5g (doesn't have to be exact, slightly more is better) succinic anhydride. Transfer to glass beaker with stirbar, move into the chemical hood. In the chemical hood, measure out 350ml of 1-methyl-2-pyrrolidinone into the remaining clean, dried 500ml cylinder. Transfer to the glass beaker with succinic anhydride, move to a stirplate, and mix. Add NaBorate as soon as succinic anhydride has dissolve, mix well, and transfer to remaining clean, dry glass chamber. Put on the lid.
16. Succinic anhydride incubation: Grab slides from centrifuge once done spinning. Check to make sure they're dry. Bring to chemical hood where

succinic anhydride solution is. Use glass dipper to transfer slides to succinic anhydride chamber. QUICKLY PLUNGE the slides into the succinic anhydride soluton, dunk several times (for about 30seconds duration), keeping the tops of the slides under the level of the succinic anhydride solution. When finished, put the lid onto the chamber, and transfer to the shaker/rotator. Incubate 15min, with gentle shaking.

17. During shaking: recycle the water and EtOH wash chambers! Rinse each out 3X with distilled H_2O, then EtOH. Re-fill each with appropriate solution, cap, and bring to chemical hood.
18. Washing succinic anhydride solution: After 15min incubation, remove the slides from the rotator. Use the glass rack dipper to pull the slides from the succinic anhydride solution and briefly drain excess succinic anhydride from the slides. Transfer slide rack to H_2O chamber, with 20-40X dunking, 1minute incubation (the purpose of this step is to remove the organic solvent). Quickly transfer the rack to the EtOH chamber, with rapid dunking.
19. Drying the arrays: Spin 600rpm in the tabletop Beckman for 3-5min. Transfer to box for storage, be sure to label as 'post-processed' and the date.

Appendix 4: Hybridisation and post-hybridization processing of micro-array slides

1. Rinse the hybridization chamber and lid with distilled water, making sure that salt crystals and other contaminants have been removed. Gloves must be worn at all times during this process. Hand oils, nucleases and other contaminants can interfere with the hybridization reaction, which proceeds in a ~10 µm layer between the microarray slide and the cover slip. After rinsing, dry the cassette chamber and lid thoroughly with paper towels.

2. Prior to use, make sure that the flexible rubber gasket is seated evenly in gasket channel. The gasket occasionally pulls free of the cassette chamber during use. If this occurs, re-insert the gasket into the gasket channel by applying light pressure. The gasket must be evenly seated in the gasket chamber to prevent leaks between the chamber and lid. Never attempt to use the hybridization cassette without a properly seated gasket.

3. Before inserting the microarray slide or substrate (25mm x 76mm x 1 mm) into the cassette chamber, add 10.0 µl of water (dH2O) to the grooves inside the cassette chamber. The water evaporates during the hybridization reaction, producing conditions of 100% humidity in the cassette chamber. A humid environment prevents evaporation of the hybridization solution between the microarray slide and cover slip during the hybridization reaction. Failure to add hybridization buffer can cause drying of the fluorescent sample onto the microarray surface, leading to elevated non-specific fluorescence.

4. After adding water into the cassette chamber, insert the microarray slide or substrate (25mm x 76mm x 1 mm) with the reactive (DNA side) of the microarray facing upwards. Make sure that the slide is seated evenly on the base of the cassette.

5. Aliquot the fluorescent sample (probe) onto the left side of a clean cover slip (0.1 mm thick) at a volume of 2.0 µl of fluorescent sample per cm2 of cover slip. The size of the cover slip should exceed the hybridization region of interest by 4 millimeters in each dimension. If the hybridization region is 1.8 cm x 3.6 cm, the corresponding cover slip should be 2.2 cm x 4.4 cm. A 2.2 cm x 4.4 cm cover slip requires 19.4 µl of hybridization solution. Standard hybridization buffers for microarray experiments contain 5X SSC and 0.2% SDS. Prior to use, cover slips should be cleaned thoroughly with mild detergent and rinsed extensively with distilled water. Cleaning is necessary to remove oils and other contaminants that are often present on commercial cover slips. After washing and rinsing, cover slips should be dried thoroughly with delicate paper wipes (e.g. Kimwipes) and inspected to make sure they are clean and free of dust and other debris.

6. To begin a hybridization reaction, gently lower the cover slip onto the microarray surface, using a pair of fine forceps. Gently pipette the sample reaction mixture from one end of the cover slip slowly and in one flow. In certain cases, small air bubbles can become trapped between the microarray slide and the cover slip. If this happens,

don't panic! Most air bubbles will exit the slide/cover slip interface following several minutes of incubation at elevated temperature.

7. The standard length of time for hybridization reactions involving >50 nucleotide probe and target pairs is 6-12 hrs at 65°C. To some extent, hybridization times and temperatures can both be adjusted for specific research applications. Once the sample is loaded onto the microarray surface, quickly place the lid on top of the chamber so that the sealing screws align with the threaded holes in the cassette base.

8. Once the lid is positioned correctly on top of the chamber base, manually tighten each of the sealing screws by applying downward pressure and turning the screws in a clockwise manner until turning becomes difficult (3-4 half turns). Check to see that the rubber sealing gasket is seated correctly in the gasket groove. If the gasket is unevenly seated, remove the lid and re-position the sealing gasket before tightening the four sealing screws.
9. After all sealing screws have been manually tightened, double check all four screws by turning each clockwise again to make sure that the cassette lid is firmly sealed against the rubber sealing gasket in the cassette base. Screws should be finger tight. Do not tighten excessively!

10. Once the hybridization chamber containing the microarray is sealed properly, submerge the cassette into a water bath incubator set at the desired temperature (65^{o}C). Once a microarray is placed inside the hybridization chamber, **do not** invert the hybridization cassette at any time before, during or after the hybridization reaction. Always keep the chamber lids facing upwards. Inverting the chamber can cause the microarray slide and cover slip to adhere to the underside of the cassette lid, leading to a loss of hybridization sample and poor results. The hybridization chambers are carefully designed to allow easy stacking. Water circulation between cassettes makes it easy to achieve uniform reaction temperatures.

11. Incubate the hybridization chamber with the lid facing upwards in the water bath incubator for the desired period of time. Typically, 6-12 hr at 37°C-65°C is suitable for most applications.

12. Following the hybridization reaction, remove the hybridization chamber from the water bath incubator and dry the outside of the cassette by blotting briefly with paper towels. Removing excess water from the outside of the cassette prevents incubator water from flowing into the cassette chamber once the sealing screws are loosened.

13. Place the hybridization chamber on the laboratory bench and manually loosen the sealing screws by turning each of them in a counterclockwise direction. When the sealing screws have been loosened completely (3-4 half turns), remove the chamber lid. Under certain circumstances, a slight vacuum will prevent the manual removal of the cassette lid. If this occurs, insert a forceps into the slot at the base of the chamber and apply gentle upward pressure.

14. Place the lid aside and remove the microarray slide from the cassette chamber. Under certain circumstances, the slide will adhere to the base of the cassette. If this happens, insert a forceps into one of the grooves in the cassette base and detach the slide from the base by applying gentle upward pressure.

15. Quickly transfer the slide to a slide rack inside a staining dish containing Wash Buffer A consisting of 1X SSC and 0.2% SDS at 25°C for 5 min. (5 min works well to remove most of the un-hybridized material). Gentle agitation of Wash Buffer A will cause the cover slip to float free from the microarray surface, 10-30 sec after submerging the microarray into the wash buffer. If the cover slip does not float free from microarray surface, gentle pressure with a fine forceps can be used to remove the cover slip. When using forceps to remove a cover slip, avoid contacting the hybridized surface directly as scratches can reduce the quality of the data.

16. Following 5 min incubation in Wash Buffer A, transfer the microarray slide to a second staining dish containing Wash Buffer B consisting of 0.1X SSC and 0.2% SDS at 25°C for 5 min.

17. After 5 min incubation in Wash Buffer B, transfer the microarray slide in to a third staining dish containing Wash Buffer C consisting of 0.1X SSC for 30 sec at 25°C to remove trace SDS. The microarray slide is then air dried and scanned.

Reagent Requirements

Hybridization buffers contain 5X SSC and 0.2% SDS.
5X SSC + 0.2% SDS

Wash Buffer A consists of 1X SSC and 0.2% SDS.
1X SSC + 0.2% SDS

Wash Buffer B consists of 0.1X SSC and 0.2% SDS.
0.1X SSC + 0.2% SDS

Wash Buffer C consists of 0.1X SSC.
0.1X SSC

Appendix 5: Microarray scanning and image analysis

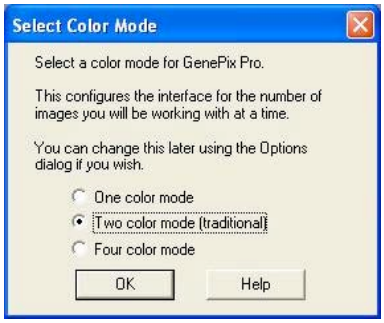
The use of image analysis software:

The ENS platform uses the GenePix Pro software. Its characteristics are available on the Axon internet site: *http://www.axon.com/ gn_GenePixSoftware.html*

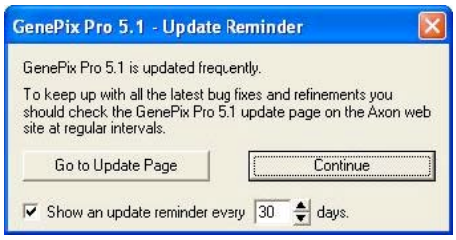
Software startup:

When GenePix Pro starts up, it is possible that some alert messages appear. We will describe some of then, so that you know what to do if they appear on screen:

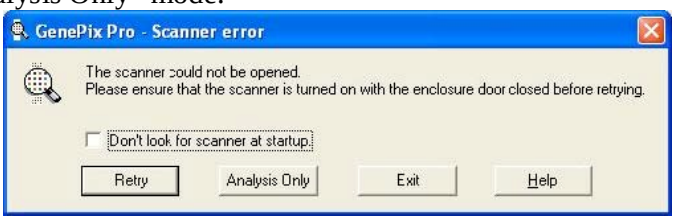
1. At the first software start-up, it can ask you in which colour mode you want to work. It depends, of course, on the way you scan your slides. In our case, we will select the "two colour" mode.



2. The update window will pop out next. It is just necessary to click on the "Continue" button.

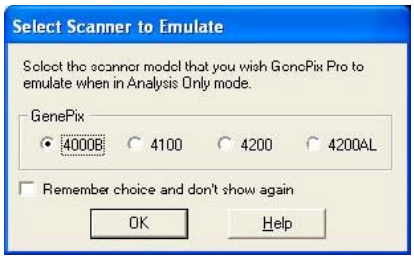


3. At start-up, GenePix Pro looks for scanners connected to the computer. If the computer you are using is not connected to a scanner, or if the scanner is switch off, select the "Analysis Only" mode:



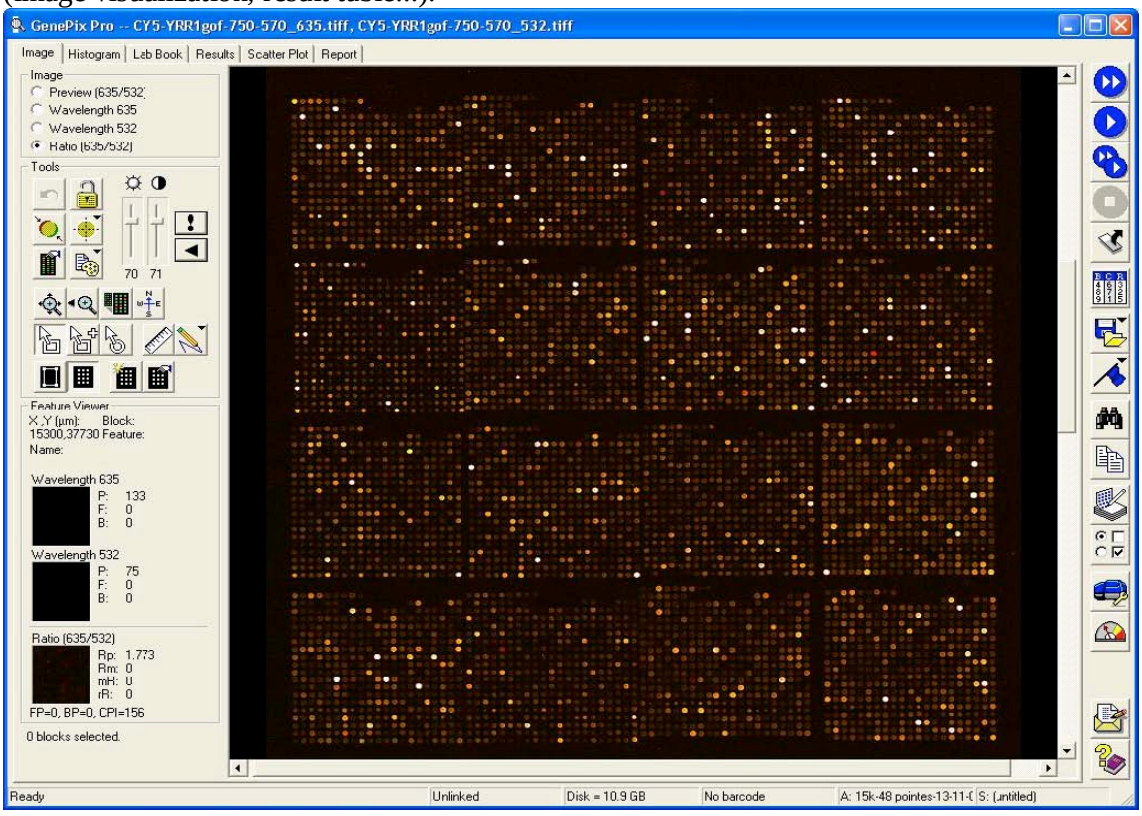
4. If not any scanner is detected; GenePix Pro will ask which scanner model you

wan to emulate. This is not so important, but it would have been preferable to choose the scanner model you use to read your slides (use 4000B as default).



An introduction to the software interface:

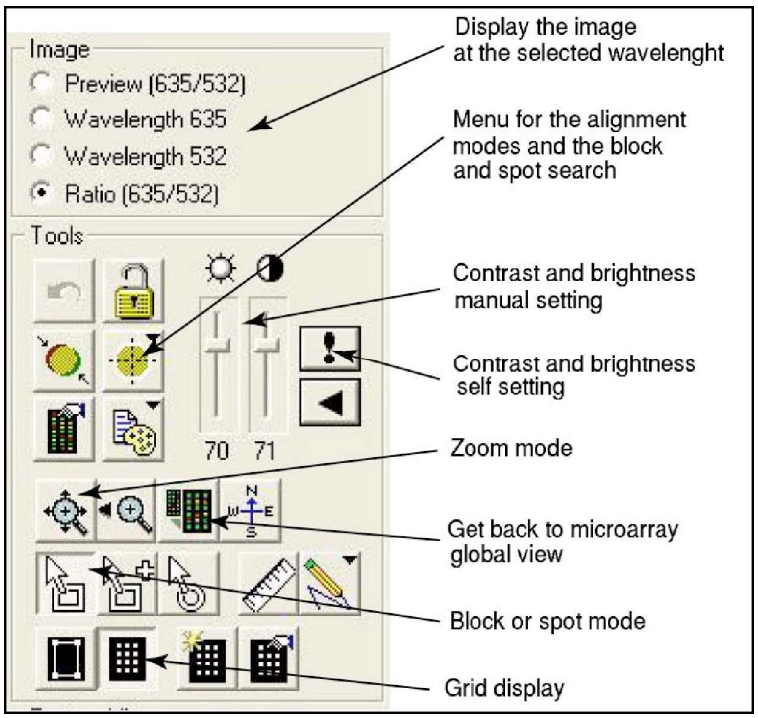
Here is an introduction to the GenePix Pro software interface (the screen shots come from the 4.1 and 5.1 software versions):
A set of tabs enables an easy navigation between the different software functions (image visualization, result table...).



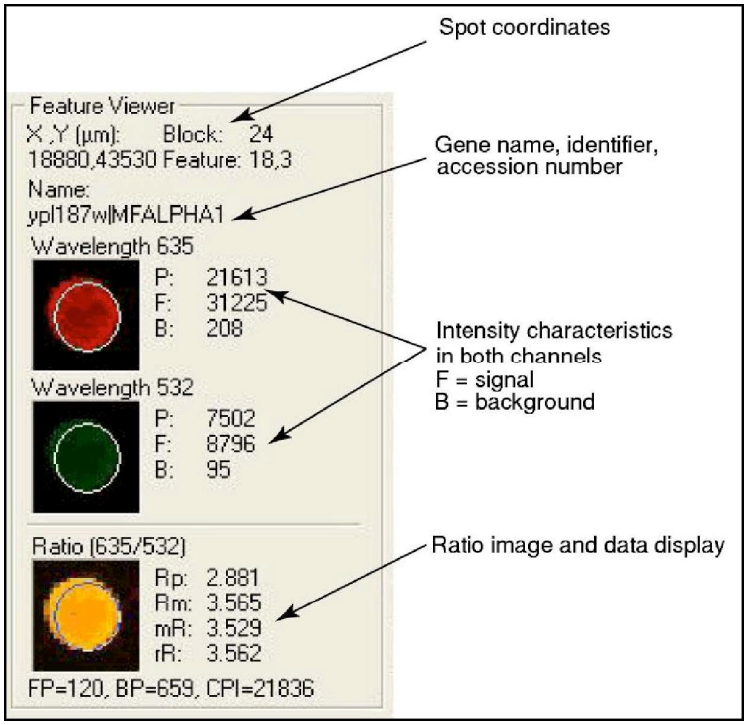
The image tab window is divided in 3 parts:

- the selection and the image navigation tools on the left
- the image visualization area in the middle
- a menu bar on the right

The toolbox on the left top of the window enables the control of the image and grid settings:



Below this, another tool enables you to visualize the different wavelength images for the spot you point out with your mouse cursor, on the microarray image. It also displays the spot intensity in each channel.



On the right part of the window, a menu bar gives access to the scanner settings and the image analysis management. We will use these following icons during the image analysis part:

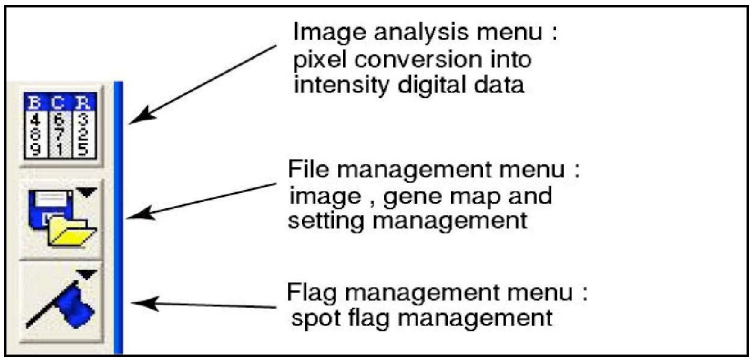


Image acquisition:

It is necessary to do a first quick reading of the slide « prescan » in order to locate the scan zone of the device.

It is important to pay attention to the gain and photomultiplicator level (PMT) choice as to big values could increase artificially the electronic noise of the device and sort out of the linearity range of the scanner. We advise to read the slide using medium PMT level (around 600-650). It could be useful to do several scans at various level of PMT in order to establish the PMT offset.

Generally, we use a pixel size of 10µm with only one scan (lines to average = 1). However, setting this value to 2 or 4 reading allow to reduce the electronic noise with a longer scanning time.

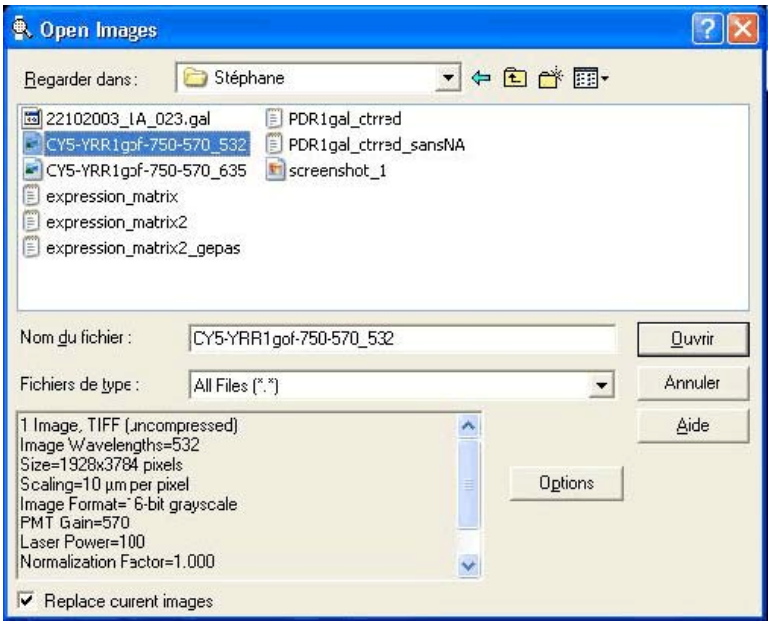
All along the scan, verify that the image is correctly equilibrated in the “histogram” panel: the two curves corresponding to the two channels must be superimposed for intensities greater than the background.

The different steps of a microarray image analysis:

Here are the practical steps to follow to achieve an image analysis thanks to the GenePix Pro software:

1. Open the image files with the file management menu icon  .The image files are in tiff format. After scanning your microarray, you can save your image as a single file (the compilation of the two wavelength images) or as a multiple file (one file for each wavelength image). The multiple file saving generates two files that are smaller than the single one and can be opened in most of the image analysis software (the single file results from the scanner software algorithm, and may not fit an other software to be opened). Be careful, we will recommend to always save your images as multiple image files, GenePix could sometimes have

a very weird behaviour using single image TIFF. If you open two TIFF images, you must select both of them at the same time in the “Open Images” window.



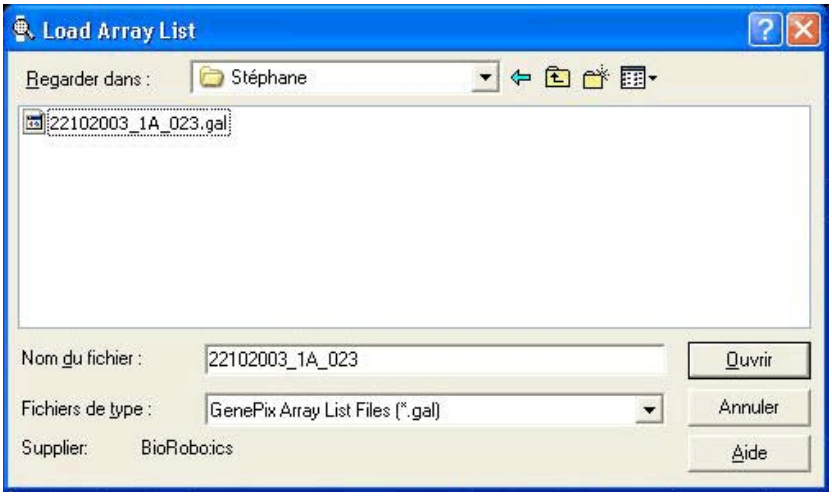
2. Now, it is useful to learn about a few buttons in the main GenePix window:

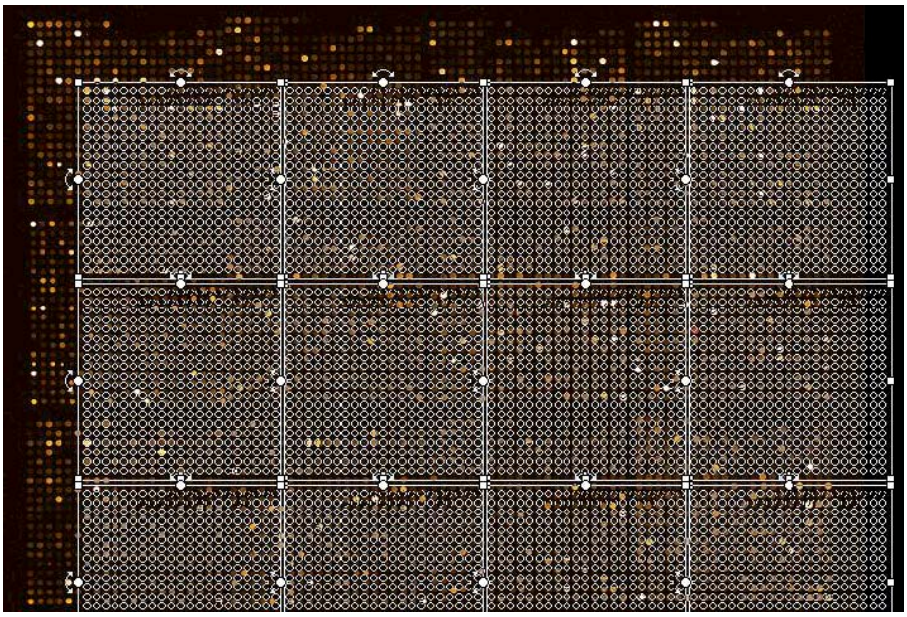
   a. First the contrast and brightness settings. These parameter modifications enable you to have a better view of your spots on the microarray image. You do not modify your images and raw data, but only what you see the computer screen.

   b. You also have to know about the zoom mode that enables you to zoom in and out to focus on a special block or spot. You can always get back to your previous zoom level.

3. After loading the images, you have to load the grid that references your image: it is the GAL format file (for Gene Array List). It contains all the informations that are needed to identify each spot (coordinates, name, identifiers) on your microarray.

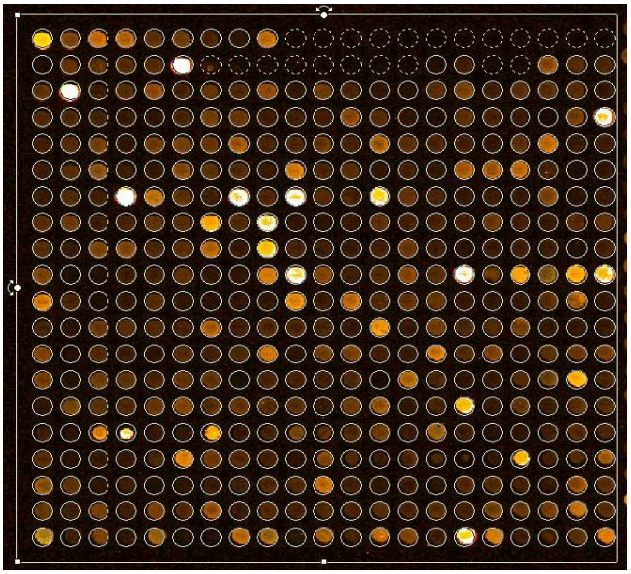
4. Use the “Load Array List” function in the file management menu  to load the grid. Be careful to always save the files using a right click on the link and using the function “Save the target as…” from the contextual menu.





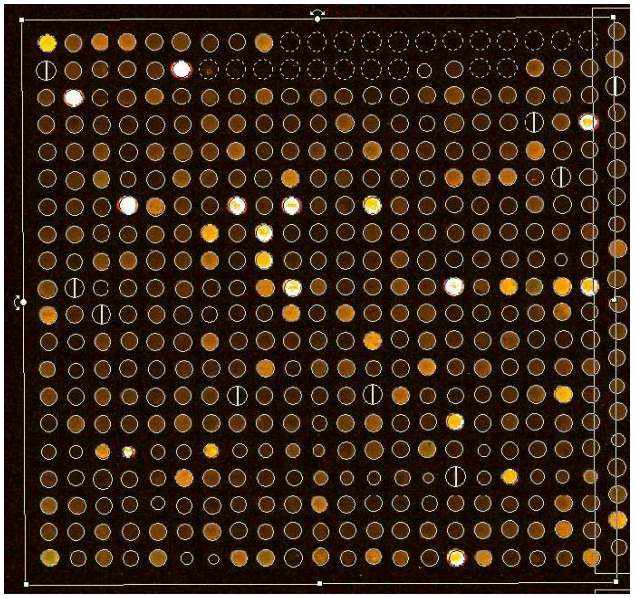
5. Once it is loaded, you have to put it properly on you image.

6. Choose the block mode  . You can now select grid blocks and move them to combine them properly with your image. The software is supposed to be able to do it automatically, but in fact, you have to give it a little help. This step is the one you should brighten and contrast the image enough to see the weak spots. Then, use the spot at the bottom left of each block to position your grid: this spot is the first one spotted on the slide. Be careful, pay attention to work in bloc mode and not in the add bloc mode (the same icon but with a plus sign).



7. Save your grid settings (use the function “Save settings” in the file management menu). Your parameters are saved in a GPS file (GenePix Settings). It is important to note that a GPS file is unique and dedicated to specific microarray.

8. Once your grid is positioned, use the “target” icon  to launch the automated identification of blocks. At the end of the process, verify GenePix identification process has worked properly to avoid errors such as mismatches on spot lines, block overlaps... To achieve this, move a single block. Next, you can launch using the same icon the spot (feature) identification inside each block.



9. Do not forget to save your grid settings regularly.

10. The next step constists in searching the spots that could be doubtful. GenePix could have considered an artefact as a spot, rejected a spot you would have accepted or mispositionned the circle around the spot. The more the image quality is high, the less this step is long.

11. Put yourself in spot (feature) mode  . You can move the grid spots inside the block, blow them up or narrow them to adapt the circle to the spots if you think they are not correct. You can also flag them if you want to filter bad spot out. This avoids you to work with spots that could be false positive such as spots with unbalanced fluorescence due to dust.

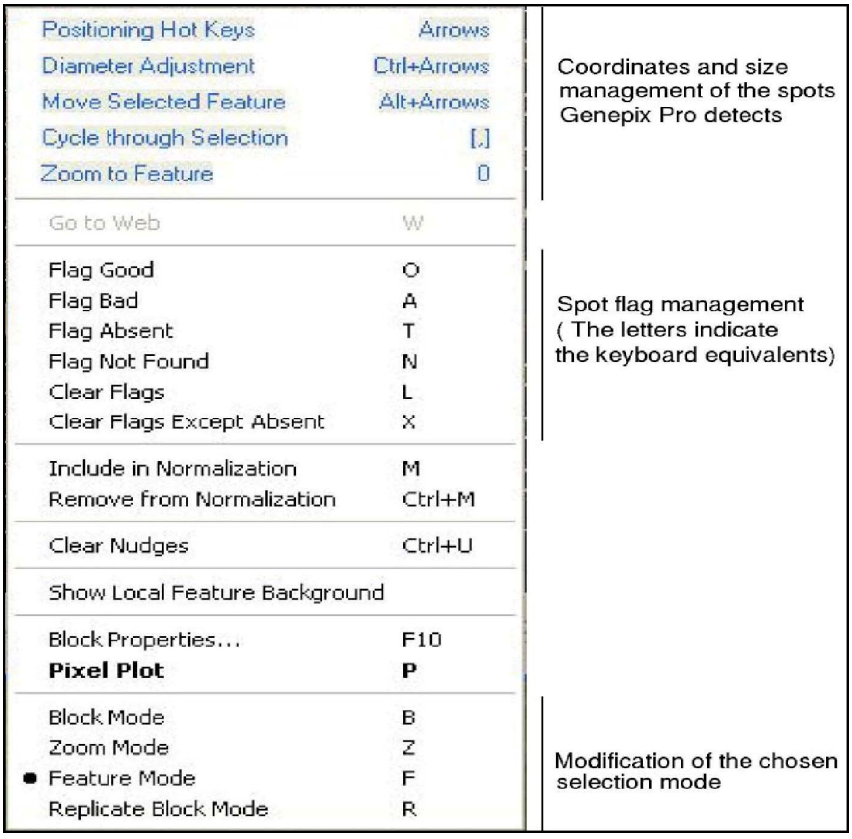
12. GenePix proposes different kinds of flags:


Absent spot (-75)

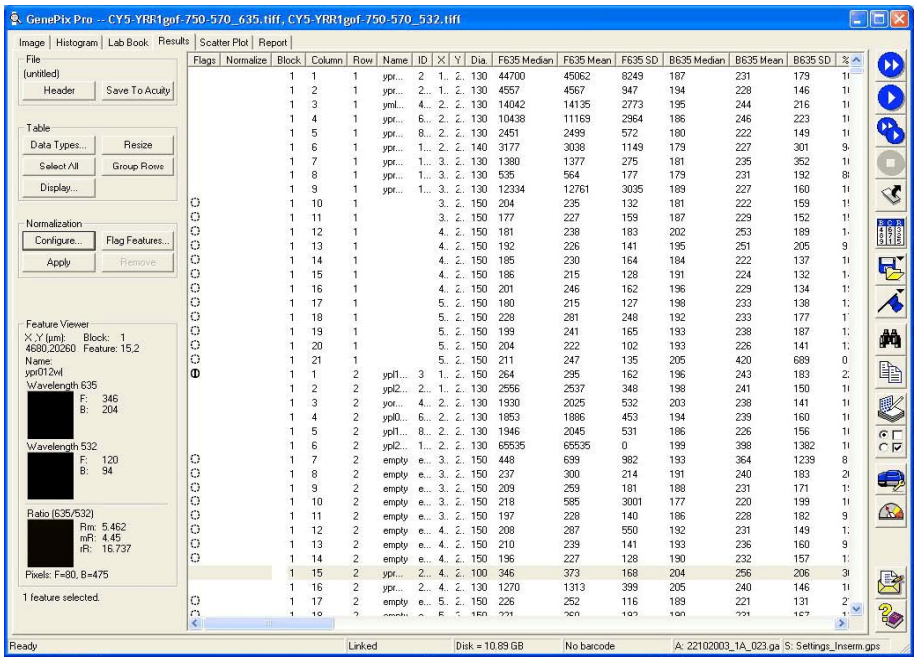

Undetected spot (-50)


Bad Spot (-100)

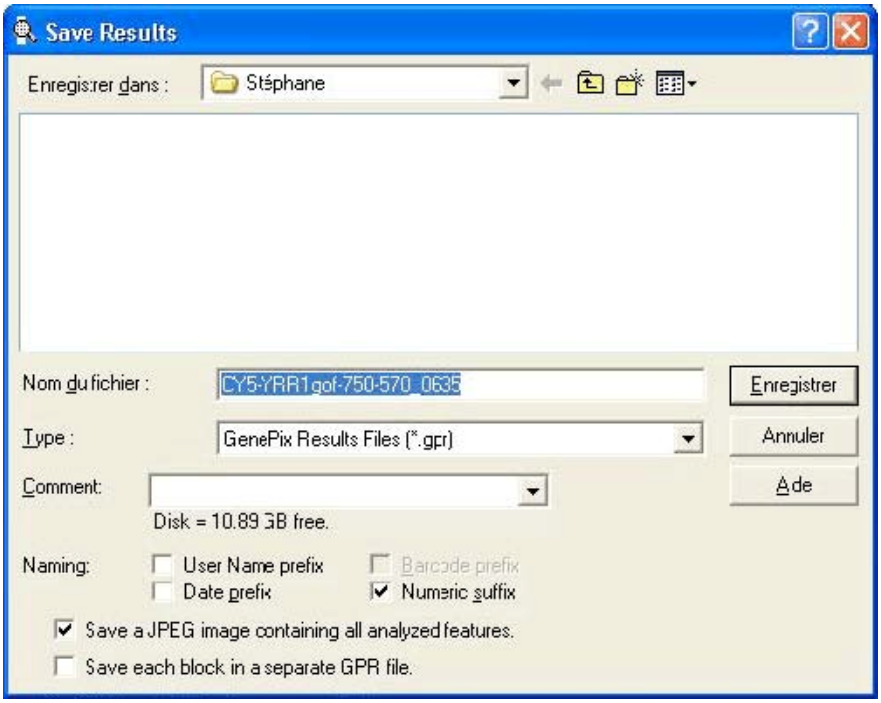
13. A right click on the spot (or a group of spots) when you work in feature mode enables you to access a pop up menu with the spot parameters. There, you can choose a flag to affect to the selected spot:



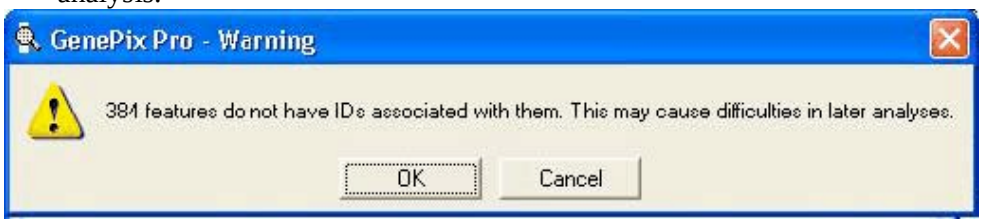
14. At the end of the spot validation, launch the analysis  that is to say the conversion of pixels into digital intensity values.



15. To get your result table, go to the result tab window and save them into a file (GPR file for GenePix Results).



16. GenePix may warn you that some spots do not have unique identifiers. In our case, it is normal because some spots are registered as “empty” in the GAL file (the grid file). Once you have saved your file, open it in a spreadsheet program such as excel to supress them so that they can not be taken into account in your analysis.



17. You are now ready to normalize your data!

Appendix 6: Whole cell PCR reactions and products cleaning

1. 96 well whole cell PCR reactions

Reagents:

Transformation plates
Multichannel pipetter
Sterile yellow tips
96 well PCR plate
lid for 96 well PCR plates

PCR mix for 100 50ul reactions:

Component	Vol/50ul rxn	Vol for 100 rxns	[final]
10X PCR buffer	5.0ul	500ul	1X
50mM $MgCl_2$	2.0ul	200ul	2mM
25mM dNTP mix	0.5ul	50ul	0.25mM
20pmole/ul M13F*	0.25ul	25ul	0.10pmol/ul
20pmole/ul M13R*	0.25ul	25ul	0.10pmol/ul
H_2O	41.8ul	4180ul	------
5U/ul rTaq	0.2ul	20ul	0.02U/ul

M13F= 5' TGTAAAACGACGACGGCCAGT 3'
M13R= 5' CACAGGAAACAGCTATGACC 3'

Procedure:

Thaw reagents for PCR mix on ice/benchtop
Make up PCR mix in 15ml tube
PCR reaction plate on ice, A1 well in upper left corner
Aliquot 50ul to each well in 96well PCR reaction plate
Use fresh box of 96 yellow tips to pick colonies and inoculate into PCR wells

Transfer to PCR machine, perform following cycles:
94C, 2min
94C, 30 sec
50C, 30sec
72C, 30sec
go to step 2, 35X
72C, 7min
4C, hold
end
(This takes about 2hrs to run)
Check yields
5ul (1/10 total) reaction + 5ul water + 2ul 6X sample buffer
1% agarose gel, use the 4x24 well comb to fit all samples

96 well PCR product purification

Reagents:
75% Isopropanol

Procedure:

Precipitation step:
Add 150ul (3 Volumes) of 75% Isopropanol to each well. No need to mix or change tips.
Seal plates with film, and incubate at room temp for 15 minutes.

Centrifuge for 30 min to precipitate:
Centrifuge 3rd floor GH, North side in JS5.3 rotor: 2800xg at 4C.
Alternatively, use Allegra 6 Centrifuge at 3000 rpm at room temperature.
Remove film, place a bunch of folded paper towels on top of tray and invert
Return inverted trays to centrifuge, spin 100xg for 2min (if using Allegra spin at 600rpm for 3 min)

Resuspension step:
Resuspend in 25ul water. No need to change tips. Spin briefly in centrifuge (2 min at 500rpm)

(Can be stored at 4 ^{o}C in the dark for 1 week or –20C in dark for up to 6months)

96 well Big Dye sequencing reactions
Reagents:
ABI 96 well plate
lid for 96 well plates
BigDye v3.1 mix for sequencing reactions:

Reagent	Volume/reaction	Volume/100rxns
Template DNA	6.5ul	-------
5X sequence rxn buffer	2ul	200ul
5-10pmole/ul primer	1ul	100ul
BigDyeMix	0.5ul	50ul

Procedure:
Make up sequencing mix
Aliquot 3.5ul sequencing mix to each well
Add 6.5ul of cleaned up whole cell PCR template to each well of sequencing reaction plate
Seal with lid
Run Sequencing PCR:

96C, 1min
96C, 10sec
50C, 5sec
60C, 4min
go to 2, (48-60cycles)
Note- increasing the cycles shown to improve sequence
10C, hold

96 well sequencing reaction clean up

Reagents:
75% Isopropanol

Procedure:

Precipitation step:
Add 40ul (4 Volumes) of 75% Isopropanol to each well. No need to mix or change tips.
Seal plates with film, and incubate at room temp for 15 minutes.

Centrifuge t for 30 min to precipitate:
Centrifuge 3rd floor GH, North side in JS5.3 rotor: 2800xg for 30 minutes at 4C.
Alternatively, use Allegra 6 Centrifuge at 3000 rpm at room temperature.

Remove film, place a bunch of folded paper towels on top of tray and invert
Return inverted trays to centrifuge, spin 100xg for 2min (if using Allegra spin at 600rpm for 3 min)

Resuspension step
Re-suspend in 10ul HiDi formamide. No need to change tips. Spin briefly (2 min at 500rpm) for better re-suspension

Optional:
Heat denature samples before loading:
96C, 1min (I've also done 95C for 2min with success)
ice 2min.