

**CHARACTERIZATION OF THE VAGINAL MICROFLORA
OF HIV POSITIVE AND HIV NEGATIVE WOMEN IN A
SUB-URBAN POPULATION OF KENYA**

Teresa Nungari Kiama, M.Sc. (Biochemistry)

A Thesis submitted for the Degree of Doctor of Philosophy
(Ph.D.) in Medical Physiology



University of Nairobi

2011

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Declaration

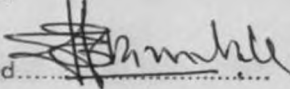
This Thesis is my original work for the degree of Doctor of Philosophy in Medical Physiology of the University of Nairobi and has not been presented for award of a degree in any other university.

Teresa Nungari Kiama, MSc.

Signed.......... Date...30.11.2011

This Thesis has been examination and submitted for publication with our approval as University Supervisors.


1. Prof. Benson Estambale, MSc, Ph.D.
Institute of Tropical and Infectious Diseases, University of Nairobi, Kenya.

Signed.......... Date...30.11.2011

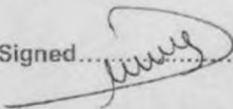
2. Prof. Paul M. Mbugua, Ph.D.
Department of Medical Physiology, University of Nairobi, Kenya.

Signed.......... Date...30.11.2011

3. Prof. Mario Vanechoutte, Ph.D.
Laboratory for Bacteriology Research, Ghent University, Belgium.

Signed.......... Date...30.11.2011

4. Prof. Marleen Temmerman, MD, Ph.D.
Department of Obstetrics & Gynaecology, Ghent University, Belgium.

Signed.......... Date...30.11.2011

DEDICATION

This Thesis is dearly dedicated:

To my Beloved Teachers and Supervisors

Who willingly share Knowledge

To my Loving Parents

Who led me to the Knowledge of God

To our Precious Children

Daniel and Gloria

Who enrich my Knowledge of God

To the Glory of God

Who is the Source of all Knowledge

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OPENING QUOTE

***I know that everything God does will
endure forever***

Nothing can be added to it

And nothing taken from it

God does it

So that men will revere Him.

(Ecclesiastes 3:14, The Holy Bible. New International Version.
1984. International Bible Society)

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APPENDICES

Closing Quote

ACRONYMS AND ABBREVIATIONS

°C- Degrees Celcius

AFLP- Amplification Fragment Length Polymorphism analysis

AIDS- Acquired immune deficiency syndrome

ARDRA- Amplified Ribosomal DNA Restriction Analysis

ART- Anti-retroviral therapy

BV- Bacterial vaginosis

BVAB- Bacterial vaginosis associated bacteria

CD₄- Cluster of differentiation 4 is a glycoprotein expressed on the surface of T helper cells

CDC- United States Centres for Disease Control

Cfu- Colony-forming unit

CI- Confidence Interval

Cm- Centimeter

CRFs- Case report forms

C_t- Threshold cycle

DGGE- Denaturing Gradient Gel Electrophoresis

DMSO- Dimethyl sulfoxide

DNA- Deoxyribonucleic acid

dNTP- dinucleotides

E. coli- Escherichia coli

EDTA- Ethylenediaminetetraacetic acid

ELISA- Enzyme-linked immunosorbent assay

et al- Scholarly abbreviation of the Latin phrase et alia, which means “and others”

FAO- Food and Agriculture Organization of the United Nations

FGT- Female genital tract

FSH- Follicle-stimulating hormone

g- Grams

h- Hour

H₂O₂- Hydrogen peroxide

HCl- Hydrochloric acid

HIV- Human immunodeficiency virus

HIV-ve- Negative for human immunodeficiency virus

HIV+ve- Positive for human immunodeficiency virus

HOCl- Hypochlorous acid

HPLC- High-performance liquid chromatography

HPV- Human papillomavirus

IUD- Intrauterine contraceptive device

KAIS- Kenya Aids Indicator Survey

KDHS- Kenya Demographic and Health Survey

Kg- Kilogram

LH- Lutenising hormone

log₁₀- Logarithm to base ten

MgCl₂- Magnesium chloride

MIS- Mucosal Immunity Study

ml- Millilitre

mm Hg- Millimetres mercury

MOH- Ministry of Health

MRS agar- Type of bacterial growth medium is so-named by its inventors: de Man, Rogosa and Sharpe, developed in 1960

NADH- Reduced form of nicotinamide adenine dinucleotide

NaOH- Sodium hydroxide

OD- Optical density

Pap- Papanicolaou smear test.

PCR- Polymerase Chain Reaction

pH- Measure of the concentration of hydrogen ions in an aqueous solution

PID- Pelvic inflammatory disease

qPCR- Quantitative/ Real-time PCR

RNA- Ribonucleic acid

Rpm- Rotations per minute

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RPR- Rapid plasma reagin	1
rRNA- Ribosomal ribonucleic acid	1
SDS- Sodium dodecyl sulfate	1
Sec- Seconds	1
SOPs- Standard operating procedures	1
Spp.- Used as a short way of saying that something applies to many species	1
STIs- Sexually transmitted infections	14
t-DNA-PCR- Intergenic spacer length polymorphism analysis	11
TDS- Tighoni Dysplasia Study	11
TPHA- Treponema pallidum haemagglutination assay	44
tRFLP- Terminal Restriction Fragment Length Polymorphism-analysis	21
UNAIDS- Joint United Nations Programme on HIV/AIDS	21
VCT- Voluntary counselling and testing centre	27
VDRL- Venereal disease research laboratory	24
VMF- Vaginal Microflora	24
VVC- Vulvovaginal candidiasis	24
WHO- World health organization	24
WSW- Women who have sex with women	24
X- Magnification	24
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ABSTRACT

Background

The vaginal microflora is dynamic and changes occur in the course of the menstrual cycle. Lactobacilli are the dominant species of vaginal microflora (VMF) in healthy women and play an important role in the maintenance of vaginal health. When lactobacilli are depleted due to antibiotic use, douching or sexual practices, other aerobic and anaerobic organisms take over, which leads to a condition known as bacterial vaginosis (BV). BV has been shown to increase the risk for the acquisition of HIV in women in sub-Saharan Africa where heterosexual transmission is the major route of infection. There is still need for more studies to fully characterize the vaginal microflora and changes occurring during the menstrual cycle. The main purpose of this study was to characterize the vaginal microflora during the menstrual cycle of HIV positive (HIV+ve) and HIV negative (HIV-ve) women with normal cervicovaginal physiology in a sub-urban population in Kenya.

Methods

Women of 18-45 years in general good health, with a regular menstrual cycle and willing and mentally competent to give informed consent were recruited in the study. Exclusion criteria were use of hormonal contraceptives, presence of sexually transmitted infections other than HIV, use of antiretroviral therapy, a CD₄ count below 250 cell/ μ l blood, pregnancy, recent delivery or abortion and previous hysterectomy or conisation.

In this descriptive comparative study 100 women (50% HIV+ve) were followed up. Of these, 74 (41 HIV+ve and 33 HIV-ve) completed the two consecutive menstrual cycle follow-up and had high vaginal swabs taken for gram stain and anaerobic culture. Sampling was done at three time points during the cycle as follows: follicular phase (days 5-8); ovulatory phase (days 12-15); luteal phase (days 19-22). Gram stains were prepared for a total of six visits for each

participant whereas anaerobic cultures were performed for a total of four visits per subject. The isolates were identified to species level by means of t-DNA PCR. For 38 women (20 HIV+ve and 18 HIV-ve) high vaginal swabs were taken for DNA extraction and quantification by real time PCR of three *Lactobacillus* spp. (*L. crispatus*, *L. jensenii* and *L. iners*) and *Gardnerella vaginalis* and *Atopobium vaginae*.

Results

Bacterial vaginosis was diagnosed at 17.5% of enrolment visits. Respectively 42.4% of HIV-ve and 31.7% of HIV+ve women harboured a normal vaginal microflora on all six visits. In 45% of both groups, at least two BV or intermediate episodes were observed. A total of 751 cultured isolates were identified belonging to 51 species. Of these, nine belonged to the genus *Lactobacillus*. In decreasing order the most common lactobacilli were *L. crispatus*, *L. iners*, *L. jensenii* and *L. vaginalis*. While 54% of HIV-ve and 60% of HIV+ve women were colonized by lactobacilli on at least one visit, respectively only 9% and 29% had lactobacilli on at least three visits.

The following species were cultured more frequently in HIV+ve women:

Lactobacillus jensenii ($p=0.01$), *L. iners* ($p=0.02$), *Gardnerella vaginalis* ($p=0.01$) and *Peptoniphilus lacrimalis* ($p=0.01$); whereas these two species occurred more frequent in HIV-ve women: *Dialister microaerophilus* ($p=0.02$) and *Streptococcus agalactiae* ($p=0.04$).

Real time PCR results show a high load of *L. iners* in the normal vaginal microflora and high levels of *L. jensenii* in half of the women with bacterial vaginosis. Both groups had a high concentration of *L. iners* and *G. vaginalis*. Although condom use was significantly higher in HIV+ve women, the number of women harboring the protective *L. crispatus* and the concentration of *L. crispatus* was remarkably lower compared to HIV-ve women.

Results based on Gram stain category show a high load of *L. iners* in the normal vaginal microflora and which increases during BV. There were high levels of *L. jensenii* in half of the women with BV while *L. crispatus* was absent in BV cases. *G. vaginalis* concentration increased progressively from normal to BV microflora while *A. vaginae* was absent in normal microflora but was detectable in intermediate gram stain and increased progressively during the BV phase.

Analysis of PCR results based on HIV status showed a high concentration of *L. iners* and *G. vaginalis* in both the HIV+ve and HIV-ve groups. *A. vaginae* was present in both groups but *L. jensenii* was not detectable in the HIV+ve women.

Analysis based on phase of menstrual cycle showed a high concentration of *L. iners* and *G. vaginalis* throughout the menstrual cycle. Concentration of *L. crispatus* increased while concentration of *A. vaginae* decreased throughout the menstrual cycle. *L. jensenii* was not detectable after the initial phase of the menstrual cycle where it was comparatively low.

Conclusions

The rate of BV was found to be equally high in both HIV+ve and HIV-ve women. Frequent fluctuations were observed in half of the women and the numbers of normal, intermediate and BV episodes were evenly spread out in the three sampling points. Minor differences in the species composition of the vaginal microflora of HIV+ve and HIV-ve women were found. *L. crispatus*, *L. iners*, *L. jensenii* and *L. vaginalis* were the predominant lactobacilli in culture. Overall the frequency of lactobacilli present in these Kenyan women was less than previously observed in similar studies of Caucasian women. *L. crispatus*, which is the predominant species in normal vaginal microflora in Caucasian women, was very low in this Kenyan study population. *L. iners* was present in high concentration in the normal vaginal microflora and increased during BV.

CHAPTER ONE

1.1 INTRODUCTION

1.1.1 The Physiology and Ecology of the Female Genital Tract

The female genital tract (FGT) can be subdivided into two physiologically distinct regions - the lower female genital tract comprising the vagina and the ectocervix and the upper genital tract comprising the endocervix, uterus, ovaries and tubae (¹Hart *et al.*, 2000). The contents of the upper genital tract are essentially sterile due in part to the action of its mucosal immune system, and which is important for the protection of the fertilised ovum. The presence of cilia and production of mucus by the epithelial cells of the cervix continually sweeps incoming organisms back down into the vagina. The mucus also contains antimicrobial substances, including lysozyme, lactoferrin, secretory leukocyte protease inhibitor, calprotectin and peptides that can either kill microbes or inhibit their growth. When conception does happen the cervical mucus thickens and forms a plug blocking the cervical opening (²Owen, 1975; ³Guyton and Hall, 2006). This mucus plug is not only a physical barrier to microbes from the lower genital tract, but also has broad anti-microbial activity that offers protection to the developing fetus. It is also active against *Streptococcus agalactiae*, the leading cause of neonatal infection (⁴Ryan and Ray, 2004).

The human lower FGT however, contains a more complex defence mechanism. The vagina is normally loaded with organisms of a commensal microflora which, in concert with the mucosal immune system, acts as the primary barrier against infections. This vaginal commensal microflora primarily consists of *Lactobacillus* species which, owing to the by-products of their metabolism, confer colonization resistance, thus protecting the vaginal epithelium from invasion by micro-organisms (⁵Wilson, 2005). This resistance is crucial since the vagina is the passageway of entry for infectious micro-organisms, including bacteria, yeasts, and viruses that are introduced through auto-inoculation or via sexual contact. The indigenous lactobacilli further protect the upper genital tract by preventing

the ascent of opportunistic and infectious micro-organisms originating from the vagina.

The vagina is a muscular, hollow tube that extends from the external vaginal opening to the cervix of the uterus. A diagrammatic representation is shown in figure 1. Its anatomical location is between the urinary bladder and the rectum. It is about three to five inches long in a grown woman. The muscular walls are lined with a stratified epithelium, which keep it protected and moist from secretions of mucous glands lining the vaginal opening (¹Hart *et al.*, 2000). Following ejaculation by male partner, sperms travel to the fallopian tubes where fertilization normally occurs. The vaginal wall is made up of several layers. The inner layer is made of vaginal rugae that permit stretching and also secrete the protective mucus. The outer muscular layer is especially important during delivery of a fetus and placenta. Among other functions the vagina also provides the route for the menstrual blood from the uterus, to exit from the body (¹Hart *et al.*, 2000). According to Wilson (⁵Wilson, 2005), between menarche and menopause, the vagina mucosa harbours stores of glycogen that can be metabolised anaerobically to release lactic and acetic acids. These acids result in an acidic vaginal medium that can inhibit the growth of many bacterial species as well as kill others. The low pH is protective against genital and urinary tract infections. The concentrations of estrogen and progesterone influence the anatomy and physiology of the vagina, and consequently the ecology of this organ. Hormonal variations during the monthly menstrual cycles of pre-menopausal women lead to changes in the vaginal microflora. The pH of the vagina also varies a great deal during the menstrual cycle. In women of reproductive age vaginal fluid between 1 and 3 grams produced daily contains carbohydrates, proteins, amino acids and other organic molecules including immunoglobulins A and G. It is a transudate deriving from vaginal tissues through gap junctions in the vaginal epithelium, and whose volume is controlled by estrogen levels. Production of vaginal fluid increases during sexual arousal as a result of increase hydrostatic pressure.

menstrual blood, which is also shed on days of menstrual cycle, is a major component of the volume of vaginal flow. One other source is a glucose- and lipid-rich fluid secreted by eccrine sweat glands, especially hair-bearing skin which may also contribute to the vaginal flow. Outside the vagina, the flow of menses is absorbed with a range of antimicrobial mechanisms, with varying effect that determine the composition of the vaginal biofilm.

The Menstrual Cycle

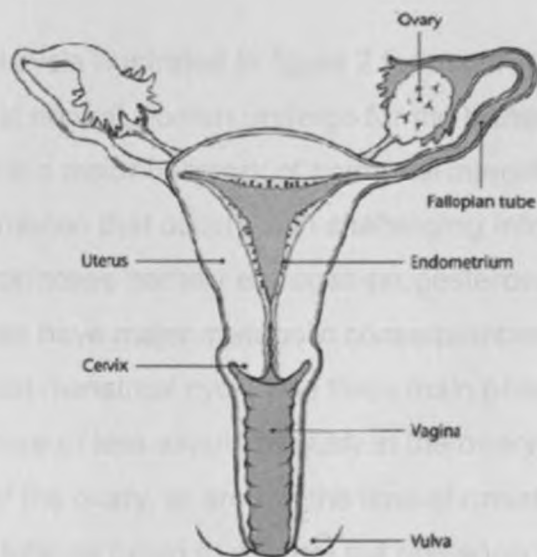


Figure 1-The female genital tract (From <http://middleeastivf.com/images/female1gbrown.gif>).

Cervical mucus, which is dependent on stage of menstrual cycle, is a major contributor to the volume of vaginal fluid, and adds mucins and proteins. Menstrual fluid provides an additional source of nutrients, especially haemoglobin from which many micro-organisms can extract iron. Despite the available supply of nutrients to microbial colonisers of the vagina, the low pH and partial pressures of oxygen, combined with a range of antimicrobial mechanisms, exert a selective effect that determines the composition of the vaginal microflora.

1.1.2 The Menstrual Cycle

The female menstrual cycle illustrated in figure 2 forms a repetitive pattern of hormonal changes that normal women undergo for the better part of their reproductive years. It is a major hallmark of hormonal integrity and a complex self-regulating phenomenon that occurs with challenging intricacy. The interplay of the major steroid hormones namely estrogen-progesterone, together with pituitary hormones, can have major metabolic consequences in the physiology of the woman. The normal menstrual cycle has three main phases, which depict changes that occur more or less simultaneously in the ovary, uterus and the vagina. At the level of the ovary, at around the time of onset of menstruation, a number of primordial follicles begin to enlarge but one soon surpasses all others and continues to grow while its rivals become atretic within a few days. This follicular enlargement continues throughout the follicular phase of the cycle, which extends for about 15 days counting from the day of onset of menses. The culmination of the follicular phase is the phenomenon of ovulation, the rupture of the follicular wall with the escape of the ovum into the peritoneal cavity. The process of ovulation is controlled by the hypothalamus of the brain which in turn regulates the release of the anterior pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (²Owen, 1975; ³Guyton and Hall, 2006).

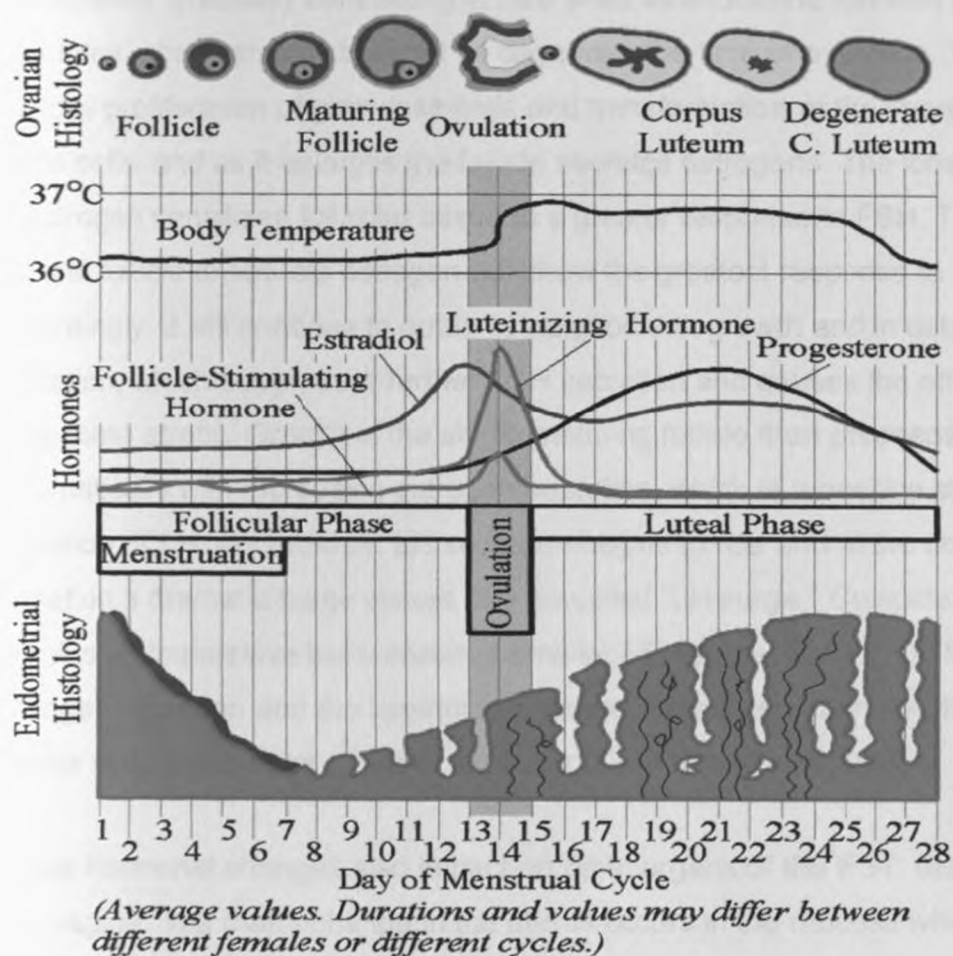


Figure 2- Changes that occur during the normal female menstrual cycle: Indicating systemic hormonal changes and histological changes in the ovary and uterine endometrium

Source: <http://www.en.wikipedia.org/wiki/Image:menstrualCycle2.png>

The ovum is attracted into the fimbriated end of the fallopian tube and begins its migration to the uterus where it is discarded with the next menstruation unless fertilized along its journey. After entering the fallopian tube, the ovum-cumulus complex is pushed along by cilia. Meanwhile, the empty follicle undergoes luteinization, gradually contracting in size while its endocrine function intensifies. This luteal phase also lasts about 14 days from the time of ovulation. The FSH induces proliferation of granulosa cells and transformation of the stroma into theca cells, and as it enlarges the follicle secretes estrogens. The local presence of estrogen sensitizes follicular tissue to a greater response to FSH. Therefore the first follicle to secrete estrogen will show the greatest response to FSH; accordingly, it will continue to outdo its neighbors in growth and in estrogen secretion, which suppresses further FSH secretion and causes the other follicles to become atretic. Growth of the single maturing follicle then proceeds automatically with increasing estrogen secretion, which is a positive stimulus to secretion of LH. Accordingly, LH secretion begins to rise and at the time of ovulation a dramatic surge occurs -the so-called "LH surge." Coincident with this event is an impressive but somewhat smaller FSH surge, a dramatic fall in estrogen secretion and the beginning of progesterone secretion. Within these events ovulation occurs (¹Hart *et al.*, 2000; ³Guyton and Hall, 2006).

These hormonal changes also impact on other organs of the FGT: uterus, cervix, and vagina. The major change in the uterus occurs in the mucosa which is a surface layer of columnar epithelial cells resting on a loose stroma of spindle-shaped connective tissue cells and blood vessels. The surface layer demonstrates numerous invaginations down into the stroma, forming tubular crypts which are loosely termed "glands". During the follicular phase, the mucosa responds to the rising estrogen secretion. The endothelial cells proliferate and the stroma becomes hyperplastic and edematous. After ovulation the predominant hormone is progesterone, and the luteal phase is associated with marked coiling of the glands and increased vascularity of the stromal layer. The individual cells show an increase in glycogen content. At the end of the luteal

phase, with falling levels of both estrogen and progesterone, menstruation begins with exfoliation of all the epithelial cells except those lining the deepest glands. Blood vessels also become necrotic with hemorrhage and sloughing of most of the stromal layer. In a normal menstrual cycle, the drop in levels of progesterone triggers menstruation. Contraction of the myometrium effects gradual cessation of bleeding and menstruation ends with a thin remnant of the stromal layer now pitted with the deepest ends of the remaining glands. The epithelium from these depths then proliferates to cover the stroma once again as the next follicular phase begins (³Guyton and Hall, 2006).

The cervix also undergoes morphological changes which are associated with those occurring in the vaginal epithelium. In the early follicular phase the endocervical mucus is scanty and viscous, and with a high protein content. As the levels of systemic estrogen rise, the cervical mucus thins out and turns watery, increasing in quantity and mucin content. As the viscosity decreases, concomitantly the elasticity of the mucus, or spinnbarkeit, increases. Spinnbarkeit is measured as the distance a strand of mucus can be stretched between slide and cover slip, which goes up to as much as 14 cm by the end of the follicular phase. The latter part of the follicular phase mucus is characterized by low levels of albumin and globulin content. It represents an infertile phase, and precedes the fertile ovulation phase (¹Hart *et al.*, 2000; ³Guyton and Hall, 2006).

In the luteal phase where progesterone is dominant, all these changes are reversed. Spinnbarkeit and ferning of vagina mucus rapidly disappear and the viscosity increases while the quantity decreases. The albumin and globulin content slowly rise as that of mucin falls. The epithelium of the vagina is stratified squamous and has been categorized into five layers. In the follicular phase, the rising levels of estrogen stimulate proliferation and maturation of all layers of the epithelium with large keratinized, acidophilic cells. In the luteal phase there is a regression and loss of the superficial layer and the cells become smaller and more basophilic while the nuclei may be larger. Polymorphonuclear leukocytes are seen in increasing concentrations just before the onset of menses.

The basal body temperature is considered to be a good sign that ovulation has occurred. It rises gradually to peak at or shortly after ovulation and persisting throughout most of the luteal phase until the onset of menses. This thermogenic response coincides with an increase of systemic progesterone to a level of 4ng/ml and continues as long as this level is maintained. Ferning and spinnbarkeit are valuable indicators when present but are not informative when absent. The ovarian steroids estrogen and progesterone have a fairly predictable effect on the endometrium, cervix, vaginal epithelium, and urethral mucosa. Further the cyclic ebb and flow of these steroids lead to diverse widespread physiologic effects on cellular metabolism. Because of the marked changes that occur during the main follicular, ovulation and luteal phases, the current study used these time point for specimen collection in the participating women (¹Hart *et al.*, 2000; ³Guyton and Hall, 2006).

1.1.3 Bacterial vaginosis

Bacterial vaginosis (BV) is reportedly one of the most common causes of vaginal infections in women (⁶Nyirjesy, 2008). This condition is characterized by a shift from the normal vaginal microflora, predominantly *Lactobacillus spp.*, to more pathogenic species such as *Atopobium vaginae*, *Gardnerella vaginalis*, *Mycoplasma hominis* and *Mobiluncus* and *Bacteroides spp.* A change in normal bacterial microflora including the reduction of lactobacilli, which may result from the use of antibiotics or certain intravaginal practices, allows more resistant bacteria to gain a foothold and multiply. In turn the latter produce toxins that affect the body's natural defenses and make re-colonization by healthy bacteria more difficult (⁵Wilson, 2005). Cases of BV are more likely to occur in sexually active premenopausal women (⁷Keane *et al.*, 1997), especially after contact with a new partner (⁸Marrazzo, 2004).

Studies show black women to have higher rates of BV compared to Caucasian populations (⁹Allsworth and Peipert, 2007; ¹⁰Royce *et al.*, 1999). Although there are no known reasons for this observation, some authors suggest vaginal hygiene practices and sexual behaviour play a role (¹¹Martin *et al.*, 1999). Some epidemiological studies of BV in African women have found possible links with HIV infection (¹²Sewankambo *et al.*, 1997). In addition, poor hygiene and low socioeconomic status are risk factors for BV (¹³Bukusi *et al.*, 2006). However, these studies were mainly conducted among either high-risk symptomatic populations or commercial sex workers. A study on Nigerian women with vaginal discharge failed to find any significant association between female genital cutting and the pattern of vaginal microflora (¹⁴Demba *et al.*, 2005). Similarly, in a separate study on well educated women with good personal care practices the authors found a higher rate of intermediate Nugent scores compared to BV (¹⁵Anukam *et al.*, 2006).

The current study investigated the cyclic variation of the vaginal microflora of HIV+ve and HIV-ve women in a sub-urban population of Kenya. We characterized the vaginal microflora in premenopausal women not taking hormonal contraceptive and free of STIs. Moreover, it is imperative to highlight the state of HIV burden in Kenya, since BV predisposes women to acquisition of HIV as well as other STIs. About 1.4 M people are infected with HIV – 7.1% of the population aged between 15 and 64 years (¹⁶KAIS, 2007). The latter survey showed a clear increase in the national prevalence of HIV from 6.7% in 2003 to the current 7.1%, with 60% of the infections being in women above 18 years of age (¹⁶KAIS, 2007; ¹⁷Kenya National Census, 1999). In addition, prevalence rates in rural Kenya rose significantly to 6.4% from the previous 5.3%, although urban prevalence rates remained higher at 8.4%. In the sub-urban Tigon area which was the study site, the prevalence rate at the time of the study was estimated at 14% (¹⁶KAIS, 2007).

Existing literature relevant to the present study has comprehensively been reviewed in chapter two of this thesis. Chapter three covers in detail the field and laboratory methods used in data collection, reported as such to accommodate the three major stages of the study, namely field methods, laboratory screening and microbiology analyses. Most of the field standard operating procedures (SOPs) and case report forms (CRFs) are retained in their original format as appendices to the relevant chapters. The results of the study are presented in chapter four and discussed in chapter five.

1.1.4 Study Rationale

1.1.4.1 Background to the Study

The rates of HIV infection in women in Kenya are significantly higher than in men (¹⁶KAIS, 2007). It has been postulated that these gender differences may be partly due to two related reasons: the impact of hormonal changes on the mucosal immunity that occur during the menstrual cycle and the concomitant increase in HIV genital tract shedding (¹⁸Al-Harhi *et al.*, 2001). An important factor determining the transmission of HIV-1 is the concentration of the virus or viral load in the female genital tract, which in turn depends to a large extent on the degree of shedding of the virus (¹⁹Critchlow and Kiviat, 1999; ²⁰Vernazza *et al.*, 1999). The higher the viral load in the female genital tract, the greater the risk that the woman will transmit the infection to partners and to children during vaginal delivery (²¹Buve, 2000; ²²Grosskurth *et al.*, 1995; ²³Quinn, 2002; ²⁴Quinn *et al.*, 2002). Therefore, better comprehension of the factors influencing the genital shedding may aid in the prevention of both horizontal and vertical transmission of the virus. At present only a limited number of studies have been carried out to define these factors. ¹⁸Al-Harhi *et al.* (2001) showed that viral shedding is elevated during and briefly after menses. This group also studied the relation of viral shedding to the pattern of the menstrual cycle and cytokine concentrations in plasma and vagina. To a limited extent, they also studied the

relation of bacterial vaginosis to viral shedding. These researchers found a positive *Candida* culture to increase interleukin-8 during menses. However, this study assessed bacterial vaginosis on the basis of the clinical Amsel criteria, which has been shown to be of limited reliability in diagnosis mainly because 40% of the women with bacterial vaginosis are clinically asymptomatic (⁸Marrazzo, 2004).

Although previous studies have followed the composition of the vaginal microflora in relation to the menstrual cycle (²⁵Eschenbach *et al.*, 2000b; ⁷Keane *et al.*, 1997), the pool of knowledge regarding vaginal microflora has increased significantly during the past few years. For instance, these studies did not differentiate between the different vaginal lactobacilli present, yet certain hydrogen peroxide-producing lactobacilli mount more colonisation resistance against invasion with anaerobes and STI pathogens (²⁶Vallor *et al.*, 2001; ²⁷Wilks *et al.*, 2004). More recently, it has been shown that *A. vaginae* is strongly associated with bacterial vaginosis, a situation that had been overlooked for a long time because this organism is extremely fastidious and was only identified by the use of molecular cloning techniques (²⁸Ferris *et al.*, 2004b; ²⁹Geissdorfer *et al.*, 2003; ³⁰Verhelst *et al.*, 2004). This serves as an indication that vaginal microflora is yet to be fully characterized, which is now possible using novel techniques.

In the present study, detailed sampling and characterization of the vaginal microflora was made possible by use of modern molecular biology techniques. The facilities at the Ghent University in Belgium were available through existing collaboration between the College of Health Sciences, University of Nairobi, and the Departments of Obstetrics & Gynecology and Microbiology at the University of Ghent, Belgium. The Ghent group was at forefront of the recent discovery of *A. vaginae* and showed that some of the vaginal microflora previously considered as normal are in fact disturbed (³⁰Verhelst *et al.*, 2004; ³¹Verhelst *et al.*, 2005). Recently published new insights into the composition of vaginal microflora

(²⁸Ferris *et al.*, 2004b; ⁸Marrazzo, 2004; ³⁰Verhelst *et al.*, 2004; ³¹Verhelst *et al.*, 2005; ³²Verstraelen *et al.*, 2004) urge researchers to reconsider existing reports on the characterization of vaginal microflora.

1.1.4 .2 Problem Statement

Since the early 1980s the world has been experiencing one of the most devastating pandemics in human history, HIV/ AIDS, a disease that continues to ravage families, communities and countries throughout the world (³³Stover *et al.*, 2002; ³⁴UNAIDS, 2004). The major hallmarks of AIDS include destruction of helper CD₄⁺ T lymphocytes and subsequent loss of immune competence. Depending on the site of initial exposure to the virus, CD₄⁺ T cells and monocytes in the blood or CD₄⁺ T cells and macrophages within mucosal tissues may be the first cells infected. The HIV, being a retrovirus (³⁵Abbas and Lichtman, 2003), integrates itself into the host cell genome, which in the case of CD₄⁺ T lymphocytes, truncates their lifespan. Because CD₄ helper cells are essential for both cell-mediated and humoral immune responses to various microbes (³⁵Abbas and Lichtman, 2003), the loss of these lymphocytes is the main reason that patients with AIDS become susceptible to many different types of infections (³⁶Weller and Williams, 2001). The development of AIDS is related to the ability of HIV to destroy the host immune system and the inability of the host immune response to eradicate HIV infection. Antibodies formed are usually virus strain-specific (³⁷Gorny *et al.*, 2005), so that antibodies from one infected individual often do not recognize HIV isolated from other infected individuals and even individual subtypes mutate quickly (³⁸Rebsamen-Waigman, 1994).

At the close of the year 2008 the number of people living with HIV/AIDS worldwide rose was about 33.4 million, with 22.4 million being in sub-Saharan Africa. In addition to 2.7 million new infections occurring annually, over 20 million have already died from AIDS, and the estimated 33 million persons now living with the disease are likely to die prematurely (³³Stover *et al.*, 2002; ³⁴UNAIDS,

2004; ³⁹UNAIDS, 2010). At least 95% of the new infections occur in countries in the developing world (³⁴UNAIDS, 2004). The WHO estimates that 70% of all AIDS cases have occurred in Africa and the highest HIV rates in the world are now in southern Africa, with a prevalence rate of about 7.5%, and more people dying of an AIDS-related illness than of any other cause (⁴⁰Lamptey, 2002; ³⁴UNAIDS, 2004). Several factors are responsible for this trend in the prevalence of HIV. Poverty, the high prevalence of other STIs, and cultural and sexual practices and beliefs all contribute to the rapid spread of HIV in Africa (⁴¹Glynn *et al.*, 2001; ⁴²WHO, 1997). This grim picture calls for pragmatic and forward-looking approaches in the prevention of the spread of HIV infection.

Figure 3 shows a comparison in percentage infection among men and women in Kenya (⁴²WHO, 1997). Current studies on HIV infection show that more women than men are infected with the disease. In an earlier survey 9% of the women tested positive compared to 5% of men (KDHS, 2004). In heterosexual transmission of HIV the woman is 2-5 times more likely to get infected than the male. In sub-Saharan Africa 60% of the HIV positive adults are women (⁴¹Glynn *et al.*, 2001; ³⁴UNAIDS, 2004). In women heterosexual transmission is the main route of infection and it accounts for more than 75% of infections worldwide (⁴⁶Rosenberg and Biggar, 1998; ⁴⁷Tarantola, 2002; ³⁴UNAIDS, 2004). Lack of availability of ART in resource poor situations means that mother-to-child transmission remains the main route of infection in children, with 50% of the infections occurring during delivery (⁴⁸John and Kreiss, 1996; ⁴⁹Quinn, 1996). The practice of unprotected sexual intercourse remains the major risk factor to acquire HIV infection in adults (²³Quinn, 2002).

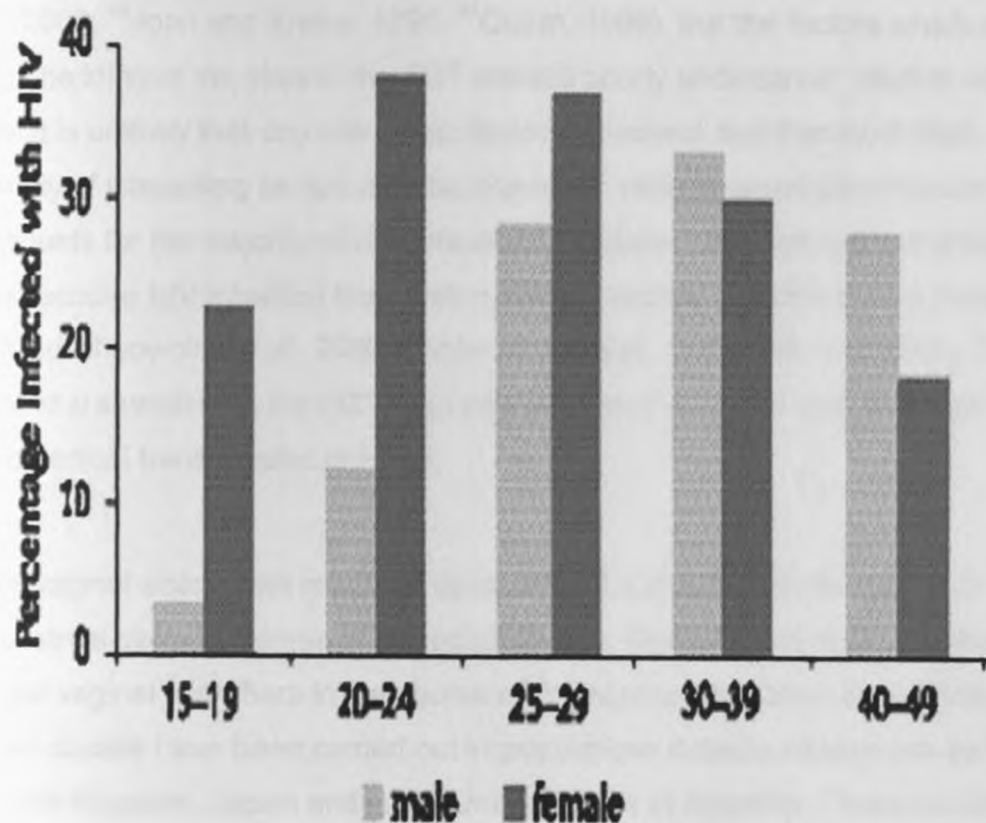


Figure 3: Pevalence of HIV in Kisumu, Kenya (WHO, 1997)

Many previous studies showed that women were simply unaware of their risk and were not taking precautions against heterosexual exposure to the HIV (³⁴UNAIDS, 2004; ³⁹UNAIDS, 2010). The principal factors influencing viral shedding in the FGT are likely to be important risk factors for both the horizontal and vertical spread of the HIV infection. More recently, the heterosexual transmission of HIV has been the subject of many studies (⁵⁰Chuachoowong *et al.*, 2000; ⁴⁸John and Kreiss, 1996; ⁴⁹Quinn, 1996), but the factors which control the shedding of the virus in the FGT are still poorly understood. Studies indicate that it is unlikely that any one single factor is involved, but that most likely a variety of interacting factors may be important. Heterosexual transmission accounts for the majority of HIV infections in adults, whereas half the children who acquire HIV infection from their mothers become infected during delivery (⁵⁰Chuachoowong *et al.*, 2000; ⁴⁸John and Kreiss, 1996; ⁴⁹Quinn, 1996). Thus, HIV viral shedding in the FGT is an important risk factor for both heterosexual and vertical transmission of HIV-1.

The vaginal ecosystem is indeed dynamic and it changes in the course of the menstrual cycle in women of reproductive age. Studies show that changes occur in the vaginal microflora in the course of the normal menstrual cycle. However, most studies have been carried out in populations outside Africa such as the United Kingdom, Japan and in the United States of America. These studies found the most profound changes in vaginal microflora to occur during menses. Only one study of African women has related the changes occurring in the VMF with cyclic menstrual changes. There is need for more studies on the VMF patterns during the menstrual cycle as we seek answers to the gaping questions on what determines the composition of VMF and to the variations that characterize the prevalence of bacterial vaginosis between women in sub-Saharan Africa and those in western countries. Follow up studies on women of reproductive age to show the short-term temporal dynamics of VMF using molecular biology methods are in short supply. Studies of VMF in African populations have mostly relied on culture-dependent techniques. There is therefore need for more studies using

quantitative PCR to map out the profiles of the main lactobacilli in reproductive age women during the phases of the menstrual cycle. Further, studies of the VMF of African women comparing HIV positive and HIV negative populations using culture-independent methods as was carried out in the current study have not been forthcoming.

The outcomes of this study will not only contribute new knowledge but also important information that will guide health policy decision in dissemination of reproductive health education to communities and especially women.

1.1.4.3 Rationale of the Study

The lactobacilli account for some 95% of the total vaginal microbial community. Under physiological conditions, the resident microflora is not exclusively *Lactobacillus* species however, and the vagina also harbours many other bacteria, albeit in relatively low numbers. These include skin microflora such as staphylococci and streptococci, enteric bacteria, as well as some typical vaginal non-*Lactobacillus* species such as *G. vaginalis*, *Prevotella* and *Peptostreptococcus* spp. The biological function of these species remains unclear. It is neither understood how these bacteria are tolerated by the resident lactobacilli nor by the host mucous immune system. Nonetheless, some typical non-*Lactobacillus* species appear to have acquired at least some degree of commensalism, making them quite often present alongside a lactobacilli-dominated microflora. The presence of these sentinel species, especially when for some reason their populations increase, has been linked to the occurrence of abnormal vaginal microflora, vaginitis, and upper genital tract infections.

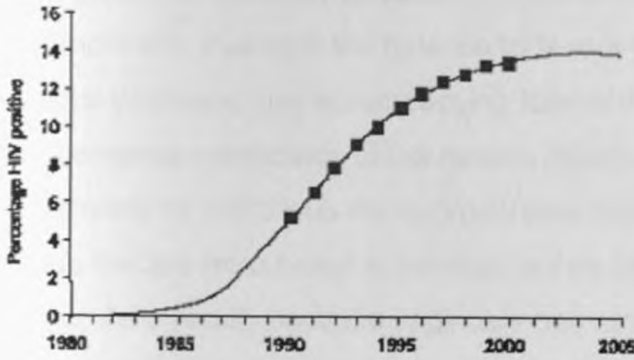


Figure 4- Adult HIV prevalence in Kenya (KAIS, 2007).

Indeed despite their intrinsic antimicrobial potential, vaginal lactobacilli often fail to maintain colonisation dominance, trading places with these sentinel bacteria

species, and leading to an overgrowth of the non-*Lactobacillus* species, giving rise to the condition of bacterial vaginosis. The initiating events to this shift remain poorly understood, as it is unclear whether the *Lactobacillus* first become depleted or less confluent, thus removing the inhibitory conditions that prevent faster growth of the other species, or whether the other species outgrow the lactobacilli and thus shift the balance to favour their own multiplication. In bacterial vaginosis, the accompanying loss of the vaginal lactobacilli equates to a near complete breakdown of the natural defence of the vagina, which is a critical port of entry for infectious micro-organisms, including bacteria, yeasts, and viruses that are introduced in the vagina through auto-inoculation or via sexual contact. As a result, bacterial vaginosis has emerged as an issue of global concern in view of its vast disease burden of infectious sequelae.

Findings show that majority of the people infected with the HIV are women; in Kenya more younger women than men are infected (⁴¹Glynn *et al.*, 2001; ⁴²WHO, 1997). The HIV infected woman can infect either her partner(s), and/ or children during delivery. In both cases infection can occur through the genital tract which means that presence of the virus in this location is crucial to its transmission. Further, the quantity of the virus present influences its transmission (¹⁹Critchlow and Kiviat, 1999). Normal vaginal microflora creates an acidic environment (⁵¹Boskey *et al.*, 1999), and lactobacilli produce hydrogen peroxide (⁵²Gupta *et al.*, 1998), both being factors that have microbicidal effects that inhibit many genital infections (⁵³Eschenbach *et al.*, 2000a), including the HIV virus *in vitro* (⁵⁴Klebanoff and Coombs, 1991) and *in vivo* (⁵⁵Hawes *et al.*, 1996; ¹¹Martin *et al.*, 1999; ¹²Sewankambo *et al.*, 1997).

The present study seeks to determine cyclic variation of the vaginal microflora of HIV positive and HIV negative women. Although a few studies have attempted to relate HIV genital shedding with resident vaginal microflora, a comprehensive analysis of this aspect is still lacking. A recent study (⁵⁶Sha *et al.*, 2005) used the clinical Amsel criteria for grading the microflora, but it is generally accepted that

up to 40% of women suffering from bacterial vaginosis remain asymptomatic (Marrazzo, 2004). However, viral load determination is outside the scope of this study. Vaginal microflora is dynamic and it changes in the course of the menstrual cycle. Factors found to have a significant influence in these changes include pregnancy, the use of medication such as antibiotics, contraception, phase of the menstrual cycle, the number of sex partners and frequency of intercourse (Wilson, 2005). In order to be able to relate HIV shedding with the changes occurring in the microflora, these factors need to be taken into consideration and study patients (volunteers) followed-up during the natural course of the menstrual cycle. Thus, a comprehensive characterization of the vaginal microflora is imperative. This is made possible by the use of modern molecular biology tools.

Efforts to find ways of preventing the spread of the HIV-1 pandemic are likely to benefit from a detailed understanding of the determinants of the cyclic variation of vaginal microflora. A comparative study like the present one should shed more light on these determinants since it will enable a comparison of similar factors in HIV infected and HIV uninfected women. This underscores the importance of studying the vaginal microflora within the natural course of the menstrual cycle as a step towards better understanding of the FGT microenvironment in sexually active women. The analysis of complex microbial communities such as those of the vagina requires a combination of culture and molecular techniques that can identify closely related species. Our study population consisted of non-pregnant premenopausal HIV+ve and HIV-ve women who were free of STIs. Known confounding host factors such as use of contraceptives and antibiotics, CD₄ counts and age were accounted for within the study inclusion criteria. We investigated variation of the vaginal microflora of HIV+ve and HIV-ve women in a sub-urban population of Kenya, and identified species present during the follicular and ovulation phases of the normal menstrual cycle.

1.1.5 Purpose of the Study

The main purpose of this study was to characterize the vaginal microflora during the menstrual cycle of HIV+ve and HIV-ve women with normal cervicovaginal physiology in a sub-urban population in Kenya.

1.1.5.1 Specific objectives

1. To identify by culture the vaginal microflora in healthy HIV+ve and healthy HIV-ve African women in the follicular and ovulation phase of the menstrual cycle.
2. To compare the identified vaginal microflora of healthy HIV+ve and healthy HIV-ve women.
3. To quantify by real time PCR *Lactobacillus* species in healthy HIV+ve and HIV-ve women.
4. To quantify by real time PCR bacterial vaginosis-associated species in healthy HIV+ve and HIV-ve women.

CHAPTER TWO

2.1 REVIEW OF THE LITERATURE

2.2 The Vaginal Microflora (VMF)

The lactobacilli that are the indigenous microflora of the vagina defend their niche by the established principle of colonisation resistance. This means that they create conditions to favour only their own existence, and which prevent invading organisms from gaining a foothold to the vaginal epithelium. This antimicrobial defence mechanism of the lactobacilli is exerted through various means. It includes the maintenance of an acidic environment due to by-products of metabolism, the production of hydrogen peroxide, exclusion from epithelial attachment sites, self- and co-aggregation, activation of the mucous immune system, the production of bacteriocins and possibly the release of biosurfactants. *Lactobacillus* is a genus of Gram-positive (stain dark blue or violet by Gram staining because of the high amount of peptidoglycan in their cell wall) facultative anaerobic bacteria. They are a major part of the lactic acid bacteria which are rod-shaped bacilli or cocci, named as such because most of its members produce lactic acid as the major metabolic end-product of carbohydrate fermentation. They are common and usually benign. In humans they are present in the vagina and the gastrointestinal tract, where they are symbiotic and make up a small portion of the gut flora. The production of lactic acid makes vaginal environment acidic, which inhibits the growth of some harmful bacteria. Their dominant presence makes the vagina naturally acidic, at pH 3.8 to 4.5 (⁵Wilson, 2005; ⁵⁷McClelland *et al.*, 2009).

Under normal physiologic conditions, the resident microflora is not exclusively *Lactobacillus* species. The vagina also harbours many other bacteria, albeit in relatively low numbers. These include skin microflora such as staphylococci and streptococci, enteric bacteria, as well as some typical vaginal non-*Lactobacillus* species including *G. vaginalis*, *Prevotella* and *Peptostreptococcus* spp. The biologic function of these species remains unclear. It is neither understood how these bacteria are tolerated by

the resident lactobacilli nor by the mucosal immune system. Nonetheless, some typical non-*Lactobacillus* species appear to have acquired at least some degree of commensalism, making them quite often present alongside a lactobacilli-dominated microflora. The main micro-organisms found in the FGT are *Lactobacillus* spp., *Staphylococcus* spp., *Corynebacterium* spp., *Streptococcus* spp., *Enterococcus* spp., *Candida albicans*, *Bifidobacterium* spp., *Gardnerella vaginalis*, *Atopobium vaginae*, *Propionibacterium* spp., Gram-positive anaerobic cocci, *Bacteroides* spp., *Porphyromonas* spp., *Prevotella* spp., *Clostridium* spp., *Fusobacterium* spp., *Veillonella* spp., *Ureoplasma* spp. and *Mycoplasma* spp. These species are also present in other body sites and cavities such as the colon, the oral cavity and skin, and where different combinations predominate respectively. *Lactobacillus* colonization is also associated with a four-fold increase in the likelihood of symptomatic vulvovaginal candidiasis (⁵Wilson, 2005; ⁵⁸Costello *et al.*, 2009; ⁵⁷McClelland *et al.*, 2009).

The presence of these sentinel species, especially when for some reason their populations increase, has been linked to the occurrence of abnormal VMF, vaginitis, and upper genital tract infections (⁵⁹Antonio *et al.*, 1999). Indeed despite their intrinsic antimicrobial potential, vaginal lactobacilli often fail to maintain colonisation dominance, trading places with these sentinel bacteria species, and leading to an overgrowth of the non-*Lactobacillus* species, a condition known as bacterial vaginosis. The initiating events to this shift remain poorly understood, as it is unclear whether the *Lactobacillus* first become depleted or less confluent, thus removing the inhibitory conditions that prevent faster growth of the other species, or whether the other species outgrow the lactobacilli and thus shift the balance to favour their own multiplication. In bacterial vaginosis, the accompanying loss of the vaginal lactobacilli equates to a near complete breakdown of the natural defence of the vagina, which is a critical port of entry for infectious micro-organisms (⁵Wilson, 2005). As a result, bacterial vaginosis has emerged as an issue of global concern in view of its vast disease burden and consequences to the health sector. Needless to say, vaginal health is critical to the reproductive health of the woman, her spouse and her yet-to-born baby, and subsequently to her community.

2.2.1 The Vaginal Ecosystem

The healthy human body harbors an extensive number of micro-organisms that inhabit surfaces and cavities exposed to or connected to the external environment. Each area of the body has its own unique collection of micro-organisms, determined by the prevailing physiologic conditions in that particular area. These micro-organisms are referred to as the "normal microflora". Those that manage to establish themselves at a particular tissue site must possess the genetic and biochemical adaptive mechanisms that guarantee their survival and reproduction under the prevailing conditions in order to qualify as the resident microflora (⁵Wilson, 2005). In health the vaginal ecosystem is stable and may harbour microflora profiles that protect against colonization by pathogenic micro-organisms. But this ability to maintain vaginal health gets interrupted every so often by among others, normal life processes such as hormonal variations, sexual intercourse and the cyclic menstrual flow. In the woman of reproductive age the primary driver of the balance of the vaginal ecosystem is the cyclic hormonal changes that begin at puberty (⁶⁰Farage *et al.*, 2009).

The female child's vagina acquires the initial colonization from the mother during parturition but this is thought to wane off with the residual maternal estrogens (⁶¹Elvik, 1990; ⁶²Farage and Maibach, 2011). The vagina is thought to have very low microbial diversity until the onset of menstrual cycle or sexual activity respectively (⁶³Farage *et al.*, 2010) when the scenario changes. In a prospective study set up to determine the differences in the presence of *G. vaginalis* in the vaginas of sexually exposed and virgin prepubertal girls, the organism was found to be significantly present in the sexually abused girls (⁶⁴Bartley *et al.*, 1987). A similar study of prepubertal girls but excluding sexually abused children did not find *G. vaginalis*, genital *Mycoplasmas*, *Trichomonas hominis*, *N. gonorrhoeae* nor *C. trachomatis* (⁶⁵Jaquier *et al.*, 1999). In a subsequent longitudinal cohort study ⁶⁶Meyn *et al.* (2002) demonstrated the acquisition of group B *Streptococcus* to be significantly associated with sexual activity. An earlier study (⁶⁷Shafer *et al.*, 1985) had also found sexually active adolescents to harbour *C. trachomatis*, *N. gonorrhoeae* and *T. vaginalis* and to have higher microorganism load compared to non-active subjects. There has also been found to be a linear correlation

between sexual activity and the occurrence of *Mycoplasmas*. The outcomes of vaginal colonization for sexually inactive adolescents resemble those of female children (⁶⁸Hammerschlag *et al.*, 1978) while those for sexually active teens are similar to adult women (⁶⁹McCormack *et al.*, 1972). Thus it can be seen that sexual activity increases the diversity of vaginal colonization.

Following the attainment of puberty the basal levels of estrogens rise subsequent to maturation of the gonads and the adrenal glands, while the hypothalamus in turn initiates the cyclic changes in levels of this hormone, which are normally associated with the female reproductive cycles (³Guyton and Hall, 2006). In the sexually mature woman the vaginal mucosa experiences cyclic increases in cellular glycogen levels especially at the ovulatory mid-phase of the menstrual cycle (⁷⁰Farage and Maibach, 2006). With the accompanying increase in the counts of lactic-producing bacteria, the production of lactic acid leads to a significant decline in the vaginal pH. The resultant vaginal milieu is thought to offer colonisation resistance that inhibits the establishment of other bacterial species (⁷¹Devillard *et al.*, 2004). The *Lactobacillus* populations in the vagina reach their peak concentration simultaneously with systemic estrogen levels (⁷²Larsen and Monif, 2001) around the ovulatory phase of the menstrual cycle (³Guyton and Hall, 2006). The presence of lactobacilli in the vagina has evolved as the criterion for definition of a healthy vaginal ecosystem, and is in fact the basis for the Nugent scoring system that is used in the diagnosis of bacteria vaginosis (⁷³Nugent *et al.*, 1991).

In the sexually mature woman several other factors contribute to dynamic changes that occur in the profile of the VMF. These factors include sexual behaviours such as sexual intercourse whereby introduction of semen alters the pH balance (⁵¹Boskey *et al.*, 1999) and nutritional factors (⁷⁴Ahluwalia and Grandjean, 2007). Others include multiple sex partners, frequency of sex, lack of condom use, contraceptive method used, douching and use of personal hygiene products, as well as STIs (⁷⁵Priestley *et al.*, 1997; ⁷⁶Smart *et al.*, 2004; ⁷⁷Hutchinson *et al.*, 2007). It is thus apparent that in a woman's lifespan from infancy to menopause, both internal and external factors hold the potential to

influence the dynamism of the VMF. In any consideration of the state of the vaginal ecosystem these factors must therefore be taken into account.

2.2.2 Vaginal Lactobacilli

The main organisms found in the FGT of healthy women are *Lactobacillus* spp. (³⁰Verhelst *et al.*, 2004). Lactobacilli are non-motile Gram-positive bacilli which are usually long and slender. So far more than 80 species have been identified, most of them microaerophilic and some obligate anaerobes, which makes this species ideally suited to colonise the vagina (⁵Wilson, 2005). The vaginal lactobacilli were originally described in the late 19th century by the German gynaecologist Albert Döderlein, who recognized their protective role in preventing other bacteria from ascending the genital tract where they caused postpartum endometritis (⁷⁸Doderlein, 1892). Named after him as Döderlein's bacilli, they were later assigned to the taxonomic class of *Lactobacillus acidophilus* (⁷⁹Thomas, 1928). Later research revealed *L. acidophilus* to be a distinct species and not a group as previously held (⁸⁰Hansen and Mocqout, 1970). In a subsequent DNA-DNA homology study, researchers showed *L. crispatus*, *L. gasseri*, *L. jensenii*, *L. fermentum*, and an unidentified species to be the dominant *Lactobacillus* species in the VMF (⁸¹Giorgi *et al.*, 1987). Studies using DNA-DNA hybridisation techniques identified six homologous species- *L. acidophilus*, *L. amylovorus*, *L. crispatus*, *L. gallinarum*, *L. gasseri* and *L. johnsonii* as constituents of the "*L. acidophilus* complex" (⁸²Fujisawa *et al.*, 1992b).

Research effort is yet to answer the pertinent question of what determines the presence of a particular *Lactobacillus* species in a woman's vagina. Of the more than 80 known *Lactobacillus* species, about 20 of them colonise the intestine. Only a few of these lactobacilli occur in the VMF where half of the women are host to those considered strong colonisers namely *L. crispatus* and *L. jensenii*, and the remaining half host the poorer colonizers, *L. gasseri* and *L. iners* (⁸³Verstraelen *et al.*, 2009). The composition of the VMF is thought to reflect the relative abundance of lactobacilli at the site of origin presumed to be the gastro-intestinal tract. One recent cross-sectional study revealed a

close correlation between the rectal and vaginal lactobacilli (⁸⁴Antonio *et al.*, 2005). The determinants of colonisation of the gut are therefore related to those of vaginal colonisation. ⁸³Verstraelen *et al.* (2009) found rectal colonisation by *L. crispatus* and *L. jensenii* to be associated with a significantly lower risk of bacterial vaginosis compared colonisation by other lactobacilli.

There has also been observed clear differences in lactobacilli colonisation with unique racial and geographical variations. Studies carried out in the US showed striking differences between black and Caucasian women. ⁸⁵Royce *et al.* (1999) found black women more likely to harbor no lactobacilli and to have pH ≥ 4.5 compared to Caucasian women. This also applied to *Mobiluncus* as well as other small gram-variable and -negative rods. A subsequent study by ⁸⁶Ravel *et al.* (2010) on the vaginal microbiome of reproductive-age women found the lack of dominance of lactobacilli to be apparently a normal occurrence among black and Hispanic women. Zhou *et al.* (⁸⁷2007; ⁸⁸2009), while studying white, black and Japanese women also came up with similar findings. ⁸⁷Zhou *et al.* (2007) found vaginal communities in which lactobacilli were not dominant to be higher in black women (33%) compared to Caucasian women (7%). Further, vaginal communities not dominated by lactobacilli had *Atopobium* and other phylotypes from the order *Clostridiales* while communities dominated by more than one species of *Lactobacillus* seldom occurred in black women, but were the norm in Caucasian women. The physiologic and genetic causes of these racial disparities in composition of VMF are not fully understood, but may partly account for known differences in the incidence and susceptibility of Caucasian and black women to bacterial vaginosis and sexually transmitted diseases (¹¹Martin *et al.*, 1999; ⁸⁶Ravel *et al.*, 2010). Further research is required to decipher the factors which determine the observed geographical and racial differences in lactobacilli colonisation, especially between African and Caucasian women.

Other studies carried out in Caucasian women using culture-dependent and -independent methods showed *L. crispatus* to be the dominant *Lactobacillus* (⁸⁹Eschenbach *et al.*, 1989; ⁹⁰Hillier *et al.*, 1992; ⁹¹Hillier, 1993; ⁵⁹Antonio *et al.*, 1999;

⁸³Verstraelen *et al.*, 2009). ⁹²Aslim and Kilic (2006) reported *L. gasseri* as the predominant strain in Turkish women while ⁹³Song *et al.* (1999) found *L. crispatus* and *L. gasseri* to be predominant in the vaginas of Japanese women. ⁹⁴Jin *et al.* (2007) also using a combination of culture and PCR techniques found *L. crispatus* to be the dominant *Lactobacillus* in both Ugandan and Korean women. However they also found unique distribution patterns of the other *Lactobacillus* in the two populations. These findings have since been contradicted by those of ⁹⁵Burton *et al.* (2003), ⁹⁶Vasquez *et al.* (2002), ⁹⁷Zhou *et al.* (2004) and more recently by the work of ⁹⁸Spear *et al.* (2011) who used molecular biology methods and found *L. iners* to be the predominant *Lactobacillus*. Since *L. iners* is easily omitted in culture studies (⁹⁹De Backer *et al.*, 2007), it has been proposed that these variations could have resulted from culture bias (⁹⁸Spear *et al.*, 2011). There is still need for follow up studies on women of reproductive age to show the short-term temporal dynamics of VMF using molecular biology methods.

Studies of VMF in African populations have mostly relied on culture-dependent techniques. Two consecutive studies carried out in Kenya showed the practice of vaginal washing to contribute to the occurrence of low concentrations of *Lactobacillus* compared to Western women (⁵⁵Hawes *et al.*, 1996; ⁸⁹Eschenbach *et al.*, 1989; ¹⁰⁰McClelland *et al.*, 2006; ⁵⁷McClelland *et al.*, 2009). Another study on *in vitro* activity of lactobacilli found *L. jensenii* to be the most predominant species (¹⁰¹Matu *et al.*, 2009). In Ugandan women ⁹⁴Jin *et al.* (2007) isolated *L. reuteri*, *L. crispatus*, *L. vaginalis* and *L. jensenii* as the predominant species. In the Gambia, West Africa, ¹⁴Demba *et al.* (2005), using the traditional culture methods, isolated *Lactobacillus* in 37.8% of the 227 women enrolled in a study of bacterial vaginosis. Using culture-independent techniques, a study of Nigerian women (¹⁵Anukam *et al.*, 2006) found *L. iners* and *L. gasseri* to be the predominant species, while a similar study in the same population found *L. iners* to be present in 64% of the samples compared to only 4.0% with *L. crispatus* (¹⁰²Anukam *et al.*, 2005). In Zimbabwe ¹⁰³De Wjgert *et al.* (2000) found that barely 46% of the women studied harbored lactobacilli in their VMF, with the absence of lactobacilli being significantly associated with HIV seropositivity. Studies of the VMF of African women

comparing HIV positive and HIV negative populations using culture-independent methods are still prominently missing in literature.

2.2.3 Antimicrobial and Antiviral effects of Lactobacilli

A VMF that is predominated by lactobacilli is not only healthy but is considered normal (¹⁰⁴Hay, 2005). Lactobacilli are known to manifest colonization resistance to prevent or reduce the likelihood of exogenous micro-organisms from becoming established in the lower genital tract of women (¹⁰⁵Sobel, 1999; ⁵Wilson, 2005). However, ¹⁰⁶Mardh and Soltesz (1983) found vaginal lactobacilli unable to inhibit anaerobic bacteria. Both mucosal barriers and innate immune factors exist that could inhibit viral infection of vaginal epithelium (¹⁰⁷Mascola *et al.*, 2000). Once lactobacilli become established at the onset of menarche, they initiate mechanisms to help maintain dominance at the vagina. It is observed that as long as this dominance persists the vaginal health is guaranteed. If for some reason the lactobacilli decrease and other species take over the vaginal metabolism, the health equilibrium is broken and the vagina becomes vulnerable to infections. Research is still ongoing to determine what breaks this equilibrium, the deficiency of lactobacilli or the overgrowth of other species (⁶³Farage *et al.*, 2010).

In order to remain dominant at the vaginal epithelium, the lactobacilli are known to employ several mechanisms geared towards their own survival and to the exclusion of other species. Some lactobacilli produce hydrogen peroxide which exerts antimicrobial effects on a wide range of competing species (²⁶Vallor *et al.*, 2001). Colonization by the hydrogen peroxide-producing lactobacilli has been shown to be protective against acquisition of herpes simplex type 2 virus and *T. vaginalis* (¹⁰⁸Baeten *et al.*, 2009) as well as *N. gonorrhoeae* (⁵⁵Hawes *et al.*, 1996). *In vitro* studies have shown *Lactobacillus acidophilus* to be virucidal to the HIV-1 virus (⁵⁴Klebanoff and Coombs, 1991), whereas a healthy vagina is protective against most sexually transmitted infections such as *T. vaginalis* and *C. trachomatis* (¹⁰⁹Kaul *et al.*, 2007). In the metabolism of lactobacilli terminal oxidation uses flavoproteins with the release of hydrogen peroxide as a by-product. Because most anaerobic bacteria lack catalase they are unable to neutralise hydrogen peroxide which is toxic to them (⁸⁹Eschenbach *et al.*, 1989). The absence of

hydrogen peroxide-producing lactobacilli was found to be a risk factor for easy colonization by anaerobes other than lactobacilli. Hydrogen peroxide-producing lactobacilli were later shown to inhibit *G. vaginalis* and *Prevotella bivia* *in vitro*. The presence of hydrogen peroxide confers broad spectrum antimicrobial activity that could jeopardise the survival of HIV-1 and possibly its transmission in the FGT (¹¹⁰Klebanoff *et al.*, 1991). The most ardent hydrogen peroxide producers are *L. crispatus* and *L. jensenii*, with this quality offering strong colonisation resistance to catalase-negative species (²⁶Vallor *et al.*, 2001). These lactobacilli have been associated with less transient VMF, thus offering greater stability than VMF dominated by *L. gasseri/iners* which was found to be more vulnerable to convert to abnormal state (⁸³Verstraelen *et al.*, 2009). There is need for studies that show the actual lactobacilli composition of normal and abnormal VMF throughout the menstrual cycle to help us draw firm conclusions on this matter.

Lactobacilli are largely responsible for the acidic vaginal environment (pH 3.8-4.5) (¹¹¹Redondo-Lopez *et al.*, 1990). It is postulated that the low pH stimulates growth of acidophilic species while suppressing non acidophilic organisms (⁸⁹Eschenbach *et al.*, 1989; ⁵¹Boskey *et al.*, 1999). The origin of the acids that induce the low pH in the vaginal mucosa is the metabolism of cellular glycogen by *Lactobacillus*, resulting in the production of lactic acid under the influence of increasing levels of systemic estrogens during the menstrual cycle (⁷²Larsen and Monif, 2001; ⁷¹Devillard *et al.*, 2004; ⁷⁰Farage and Maibach, 2006; ³Guyton and Hall, 2006). There are about 10^8 lactobacilli in a healthy vagina that account for the low pH as well as re-acidification following sexual intercourse (⁵¹Boskey *et al.*, 1999). It has been shown that hormone replacement therapy in menopausal women restores the low vaginal pH with subsequent *Lactobacillus* colonisation (¹¹²Galhardo *et al.*, 2006; ¹¹³Gupta *et al.*, 2006). The capacity of lactobacilli to inhibit the growth of many pathogens has been demonstrated *in vitro*, and forms the basis of the use of probiotics to treat malfunctions of the gastrointestinal and vaginal tracts. In a recent study (¹⁰¹Matu *et al.*, 2009) designed to assess the ability of lactobacilli to inhibit vaginal microorganisms usually linked to the development of bacterial vaginosis, evidence was adduced for the inhibition of *Bacteroides fragilis*,

Prevotella bivia, *G. vaginalis* and *Mobiluncus* spp. In culture, the acidity of lactobacilli supernatants determined their inhibitory potential. Further, it was demonstrated that the acid, bacteriocins and hydrogen peroxide produced by lactobacilli augment each other in their activity against the pathogens.

The antimicrobial activity of lactobacilli can further be attributed to the production of several organic acids, among them being bacteriocins, proteinaceous compounds which inhibit the growth of related species (¹¹⁴Holzappel *et al.*, 1995; ¹¹⁵Delves-Broughton *et al.*, 1996). Bacteriocins from lactic acid bacteria have been applied as preservatives in the dairy industry and are digested by proteases of the digestive tract. Bacteriocins exert bactericidal action due to their ability to disrupt the membranes of other cells, thus interfering with their function (¹¹⁶Karaoulu *et al.*, 2003; ¹¹⁷Jagadeeswari *et al.*, 2010). They have also been found to be sensitive to high pH and resistant to catalase (¹¹⁶Karaoulu *et al.*, 2003) and to lose their activity at high pH (¹¹⁸Barefoot and Klaenhammer, 1984; ¹¹⁹Diaz *et al.*, 1993). ¹²⁰Robredo and Torres (2000) found *L. salivarius* bacteriocins to strongly inhibit the growth of isolates of *Staphylococcus aureus* and *S. epidermidis*. Vaginal lactobacilli have in turn been found to produce bacteriocins with bactericidal activity against vaginal *L. gasseri*, *L. acidophilus*, strains of *G. vaginalis* and of *Pseudomonas aeruginosa* (¹¹⁶Karaoulu *et al.*, 2003). From the foregoing reports it is evident that the low pH of the vaginal ecosystem favours the bactericidal activity of *Lactobacillus* species.

Lactobacilli are also known to produce mucins, biosurfactants and collagen binding proteins as mechanisms of maintaining colonisation dominance in the vagina (¹⁰⁴Hay, 2005). A collagen-binding protein previously purified from a strain of *L. reuteri* (¹²¹Aleljung *et al.*, 1994) has also been found in *L. fermentum* RC-14 displaying anti-adhesive properties against *Enterococcus faecalis* (¹²²Heinemann *et al.*, 2000). It has been suggested that lactobacilli likely bind to extracellular matrix proteins in order to gain adherence to host tissues, and that release of these binding proteins may in turn

contribute to the ability of lactobacilli to wade off invading pathogens (¹²²Heinemann *et al.*, 2000) by obstructing available binding sites. It can be deduced therefore, that lactobacilli employ a combination of antimicrobial and antiviral effect as well as surface properties as mechanisms of colonisation dominance within the gastrointestinal and urogenital tracts.

2.2.4 Estrogen-dependent *Lactobacillus* Colonisation

During embryonic development the vagina derives from the mesoderm (⁷⁰Farage and Maibach 2006). Infant vaginal cells respond to residual maternal estrogen, an effect that wanes off by the first month, and subsequent to which the girl's body initiates estrogen synthesis (¹²³Bernbaum *et al.*, 2008). At menarche the vagina reaches maturity and acquires secondary sex characteristics subsequent to the functional maturation of the gonads and adrenal glands, setting the stage for initiation the "seed" lactobacilli to multiply. During the woman's reproductive years this organ is responsive to the cyclic rise-and-fall of estrogens, with the greatest mucosal thickness and cellular glycogen levels coinciding with the ovulatory phase of the menstrual cycle (¹²⁴Forsum *et al.*, 2005; ⁷⁰Farage and Maibach 2006). With the rising levels of estrogen, there is a concomitant increase in the populations of lactic-producing bacteria, which leads to a significant decline in the vaginal pH. This results in a vaginal environment that favours *Lactobacillus* establishment against other bacterial species (⁷¹Devillard *et al.*, 2004). Vaginal lactobacilli populations reach their peak simultaneously with systemic estrogen levels (⁷²Larsen and Monif, 2001) around the ovulatory phase of the menstrual cycle. The vagina also undergoes physiological adaption to meet the demands of pregnancy and parturition, and the onset of menopause sets in motion tissue atrophy (³Guyton and Hall, 2006).

It has been observed that lactobacilli are absent before menarche and that their numbers tend to decline with age (¹²⁵Fujisawa *et al.*, 1992a). The growth of vaginal lactobacilli is particularly challenged during the premenstrual period when the levels of plasma estrogen dip (³Guyton and Hall, 2006). This situation is reversed to a maximally

favourite one at the height of systemic estrogen release when growth, persistence and antimicrobial expression of the vaginal lactobacilli occurs. The VMF is most unstable during the week of menses (⁷Keane *et al.*, 1997; ¹²⁶Morison *et al.*, 2005). The tidal waves of circulating estrogen and progesterone levels during the menstrual cycle signal the most profound changes in vaginal environment. Estrogen influences several events at the vaginal mucosa. These include the expression of cell surface receptors, the amount and viscosity of cervical mucus, the amount of vaginal transudate, levels of glycogen, vaginal oxygen and carbon dioxide tension, reduction-oxidation potential and pH (¹²⁷Wagner and Ottesen, 1982; ¹⁰⁴Hay, 2005). The repeated cycles of estrogen level affect the confluence of the vaginal lactobacilli and consequently their colonisation resistance to external invasion. During menses the vaginal pH may rise to almost neutral (¹²⁷Wagner and Ottesen, 1982), a condition that weakens epithelial adherence of the lactobacilli and increases competition by infectious micro-organisms (¹²⁸Catalanotti *et al.*, 1994). Studies have indeed shown that *Lactobacillus* growth increases over the menstrual cycle, whereas the concentration of non-*Lactobacillus* species tends to be higher at menses, which is evidence that the VMF becomes less stable at this time (¹²⁹Eschenbach *et al.*, 2000b; ⁷Keane *et al.*, 1997; ¹²⁶Morison *et al.*, 2005). In addition, in women with recurrent bacterial vaginosis, a disease episode was shown to arise most often around the time of menstruation and to resolve spontaneously in mid-cycle (¹³⁰Hay *et al.*, 1997). It may be inferred that the estrogen-dependent lactobacilli-driven colonisation resistance has evolved as a mechanism that serves human reproduction during reproductive life span.

The vaginal ecosystem is indeed dynamic and it changes in the course of the menstrual cycle in women of reproductive age (¹³¹Owen, 1975; ⁶⁰Farage *et al.*, 2009). Studies show that changes occur in the vaginal microflora in the course of the normal menstrual cycle. However, most studies have been carried out in populations outside Africa such as the United Kingdom (⁷Keane *et al.*, 1997; ¹³²Wilks and Tabaqchali, 1987), Japan (¹²⁵Fujisawa *et al.*, 1992a) and in the United States of America (¹²⁹Eschenbach *et al.*, 2000b; ¹³³Ness *et al.*, 2006; ¹³⁴Onderdonk *et al.*, 1986; ¹³⁵Schwebke *et al.*, 1999). These studies found the most profound changes in vaginal microflora to occur during menses.

Only one study of African women has related the changes occurring in the VMF with cyclic menstrual changes (¹²⁶Morison *et al.*, 2005). There is need for more studies on the VMF patterns during the menstrual cycle as we seek answers to the gaping questions on what determines the composition of VMF and to the variations that characterize the prevalence of bacterial vaginosis between women in sub-Saharan Africa and those in western countries. It is postulated that genetic make up/ race predetermines the composition of the VMF (⁸⁷Zhou *et al.*, 2007) although no tangible evidence is available this far.

2.2.5 Host Factors Challenging Lactobacilli

The healthy human vagina is defined by the extent to which lactobacilli have succeeded to exclude other micro-organisms and taken predominance at this site of colonisation. The ability to do this ensues from a combination of factors which favour lactobacilli against other species. As previously expounded, it is the ability to put up strong colonisation resistance via known mechanisms such as maintenance of low pH and production of hydrogen peroxide and of various antimicrobial substances, which prevent competition for available attachment sites and facilitate adherence of lactobacilli (⁵Wilson, 2005). Even in health the stability of the vaginal ecosystem gets regular assaults from host factors such as cyclic menstrual flow, hormone fluctuations and the sexual act (⁶⁰Farage *et al.*, 2009) and other sexual behaviours (¹³⁵Schwebke *et al.*, 1999; ⁷⁶Smart *et al.*, 2004) as well as douching (⁵⁵Hawes *et al.*, 1996; ¹³⁶Brotman *et al.*, 2008a).

About 78% of women do not have the 'healthy' lactobacilli-dominated VMF throughout their menstrual cycle, even though they show no signs of vaginal infection (⁸Marrazzo, 2004; ⁵Wilson, 2005). According to ⁵Wilson (2005), the composition of the VMF is markedly affected by a number of host factors that include genital infections, the method of contraception, sexual activity and maturity of the individual, pregnancy and antibiotic treatment. Previous studies have found the production of hydrogen peroxide to be associated with persistent colonization. ²⁶Vallor *et al.* (2001) found that about 85% of the women who demonstrated persistent colonization harbored hydrogen peroxide

producing strains of *L. crispatus* and *L. jensenii*. In contrast, women colonised by strains of the same species but which did not produce hydrogen peroxide lost colonization after the first study visit. *L. jensenii* and *L. gasseri/iners* have been associated with weak colonisation resistance compared to *L. crispatus* which offers more stability, with the former holding a much higher risk of transition to abnormal VMF (⁸³Verstraelen *et al.*, 2009). ¹³⁷Kalra *et al.* (2007) suggest that following recovery from a bout of bacterial vaginosis, patients recolonised by *L. iners* stood a higher risk of disease recurrence.

Of the several factors identified that can alter the healthy vaginal ecosystem, the menstrual flow is the most profound of them. The rhythmic pattern of protein and steroid hormones that is characteristic of the menstrual cycle results in alternating periods of very low and high estrogens (¹³¹Owen, 1975), the main hormone that has effects on the vaginal epithelium. As the level of estrogen dips during menses, the glycogen content of the vaginal epithelial cells falls and with this is a concomittant rise in pH, thus removing the environmental conditions that favour lactobacilli (⁶⁰Farage *et al.*, 2009). The resulting conditions allow for growth of other species, usually present in low quantities, to flourish as the inhibiting acidity and other associated deterrents are no longer present, a situation also present in menopause (¹²⁵Fujisawa *et al.*, 1992a). Studies show the VMF to be most unstable in the follicular phase when estrogen levels are lowest (⁷Keane *et al.*, 1997; ¹³⁴Onderdonk *et al.*, 1986; ²⁵Eschenbach *et al.*, 2000; ¹²⁶Morison *et al.*, 2005). Further, the perceived benefits of high estrogen are supported by the observation that the prevalence of BV decreases during pregnancy (¹³⁸Yen *et al.*, 2003) and in women who use the combined oral contraceptive (¹³⁹Hay *et al.*, 1994; ¹⁴⁰Riggs *et al.*, 2007). Recently, using quantitative PCR, ¹⁴¹Srinivasan *et al.* (2010) observed menses to be accompanied by an increase in the level of *G. vaginalis* and by a decrease in *L. jensenii* and *L. crispatus*. These researchers observed that at the end of menstruation, quantities of *G.vaginalis* dropped below detection limits of this test but those of *L. crispatus* and *L. jensenii* recovered while the level of *L. iners* dropped. There is need for more studies using quantitative PCR to map out the profiles of the main lactobacilli in reproductive age women during the phases of the menstrual cycle.

The benefits of low pH and production of hydrogen peroxide and of various antimicrobial substances are lost due to certain sexual behaviours. Sexual behaviors linked to causation of inequilibrium in the VMF resemble those found to be associated with bacterial vaginosis (¹³⁵Schwebke *et al.*, 1999). The frequency of sexual intercourse as well as increased number of partners has been demonstrated to result in altered VMF (¹³⁵Schwebke *et al.*, 1999; ²⁶Vallor *et al.*, 2001). Women who had frequent vaginal intercourse exceeding one episode per week stood a higher risk losing the hydrogen peroxide producing lactobacilli. Similar results were obtained for subjects on antibiotic treatment (²⁶Vallor *et al.*, 2001). Infrequent condom use, as well as the application of vaginal medication and spermicides has also been associated with adverse changes in the VMF (¹³⁵Schwebke *et al.*, 1999). A recent study also found unprotected sexual activity to be an important factor in the deviation from normal to abnormal VMF (⁷⁷Hutchinson *et al.*, 2007).

Other host-related factors that impact on the survival of vaginal lactobacilli emanate from infections at this site. Genital infections can influence the composition of the VMF (¹³⁹Martius *et al.*, 1988; ⁵⁵Hawes *et al.*, 1996). ²⁶Vallor *et al.* (2001) found the capacity of lactobacilli to produce hydrogen peroxide to be a determinant of colonisation resistance. This implies that invading microorganisms would find it more difficult to lodge in this "unfriendly" environment. Since hydrogen peroxide blocks adherence sites from invaders, it protects from genital infections such as HIV and *N. gonorrhoeae* (⁵⁵Hawes *et al.*, 1996; ¹²Sewankambo *et al.*, 1997; ¹¹Martin *et al.*, 1999). When the equilibrium of the vaginal ecosystem is functional, it is known to inhibit the transmission of STIs (¹⁴⁰Newton *et al.*, 2001). The distortion of this balance is associated with an increased risk of HIV infection while a "normal" VMF reduces the likelihood of heterosexual transmission of HIV (¹⁴¹Schwebke, 2001). Further, Studies show that most contraceptive methods in use today, apart from the condom, have the potential to alter the composition of the VMF, usually with adverse consequences (⁵⁵Hawes *et al.*, 1996; ⁵³Eschenbach *et al.*, 2000a; ⁵Wilson, 2005). In addition oral contraceptives cause some degree of FGT inflammation (¹⁴²Geisler *et al.*, 2004).

2.3 Bacterial Vaginosis

2.3.1 Epidemiology of Bacterial Vaginosis

Bacterial vaginosis (BV) is a universal problem. It is a remarkably prevalent condition, diagnosed in 40-50% of women presenting with vaginitis (¹⁴³Spiegel, 2002). As a clinical entity BV was first described in the 1950s (¹⁴⁴Gardner and Dukes, 1955). These researchers described it as a non specific vaginitis associated with discharge, increased pH, odour and microscopic evidence of squamous cells, to distinguish it from specific vaginitis caused by yeast and *Trichomonas*. Further, they consistently isolated a Gram positive bacillus, which they later named *Haemophilus vaginalis*. The infective nature of this organism was demonstrated. However with passage of time, coupled with increased research interest in this field, the epidemiology and clinical features of BV have now clearly been described. In the recent evaluation of women aged 14 – 49 years, the National Health and Nutrition Examination Survey found 29% of them were positive for BV and the prevalence was about three times greater in African American women (⁹Allsworth and Peipert, 2007).

The condition known today as BV has undergone several changes of its name since the initial description. *Haemophilus vaginalis*, now known as *Gardnerella vaginalis* and affiliated to the family *Bifidobacteriaceae* (¹⁴⁵Catlin, 1992; ¹⁴⁶Greenwood and Pickett, 1979; ¹⁴⁷Piot *et al.*, 1980) was singled out the aetiological agent of non-specific vaginitis. The organism was isolated from the vaginas of 92% of patients with non specific vaginitis, (¹⁴⁴Gardner and Dukes, 1955), prompting the authors to change the name of the condition to *Haemophilus vaginalis* vaginitis. The term 'vaginosis' was applied to accommodate the observation that there occurred increased discharge without the accompanying inflammation usually found in specific vaginitis (¹⁴⁸Holmes *et al.*, 1981). The name 'bacterial vaginosis' was adopted since only bacterial species were associated with this, even though their identification would await a later date. The majority of bacteria were anaerobic, prompting some authors to use the term 'anaerobic vaginosis' (¹⁴⁹Blackwell and Barlow, 1982). In the late 1980s, the name 'vaginal bacteriosis' was recommended as describing the condition better (¹⁵⁰Hill and Embil, 1986; ¹⁵¹Huth, 1989; ¹⁵²Sobel, 1989). However, use of the term "bacterial vaginosis" has

persisted to date.

Several attempts have been made to describe the disease pattern of BV. The majority of published studies on the prevalence of this condition report centre-based estimates obtained from systematic screening of patients attending gynaecology related clinics. Prevalence estimate reports from European and American studies place the range between 4.9% and 36% (¹⁵³Morris *et al.*, 2001b). Yet it has recently been asserted that BV is the commonest cause of vaginitis and the reason for which women seek medical attention (⁶Nyirjesy, 2008). A recent population-based survey in America (*National Health and Nutrition Examination Survey 2001-2004*) placed the prevalence of BV at 29.2% in the total population. Racial discrepancies arose with a prevalence of 51.4% and 31.9% among African and Mexican Americans respectively, while among Caucasian women it was 23.2% (¹⁵⁴Koumans *et al.*, 2007). In a community-based prospective cohort study carried out in the UK, a prevalence of 14.5% was adduced (¹⁵⁵Ugwumadu *et al.*, 2003). It is thus apparent that the prevalence of BV may vary between the races. A community-based study in rural Uganda found the prevalence to be 50% (¹²Sewankambo *et al.*, 1997; ¹⁵⁶Wawer *et al.*, 1998), similar to what is observed with African American women. Whether within a normal or abnormal situation, the VMF is substantially heterogeneous (¹⁵⁷Tohill *et al.*, 2004). It is however not clear whether differences in the prevalence rates reported reflect geographical variations in species carriage.

Most studies carried out among African women have focused on the sexual practices that contribute to the alteration of the VMF, leading to the development of BV, and with particular focus on HIV infectivity. The prevalence of BV has been shown to be highest among African women (¹⁰Royce *et al.*, 1999). A closer look at existing studies illustrates this assertion. In investigating the genitourinary infections of women of reproductive age in Burkina Faso, ¹⁵⁸Ledru *et al.* (1996) found the prevalence of BV vaginosis to be 19.7%. In the Gambia, ¹⁴Demba *et al.* (2005), investigating hygiene practices and VMF of women presenting with vaginal discharge, confirmed the high rates of BV in African women. The Nugent score (⁷³Nugent *et al.*, 1991) and the Amsel criteria (¹⁶³Amsel *et*

al., 1983) methods yielded prevalences of 47.6% and 30.8% respectively. This study found a significant association between BV and *G. vaginalis*, *Mycoplasma hominis*, *Ureaplasma urealyticum* and *Bacteroides*, *Mobiluncus*, *Peptostreptococcus*, *Porphyromonas* and *Prevotella* spp. While investigating the association of abnormal VMF with HIV-1 infection, ¹²Sewankambo and colleagues (1997) found the rates of BV to be as high as 50% in women with and without symptoms. Infection with HIV-1 was significantly higher among women with BV. A cohort study of pregnant women in Malawi (¹⁶⁰Taha et al., 1999) found the prevalence of BV to be 30%. In a separate study of pregnant and postnatal women in Malawi (¹⁶¹Taha et al., 1998a), disturbances of VMF were shown to increase HIV. The researchers concluded that BV could increase acquisition of HIV among women of reproductive age.

In Nigeria an earlier study (¹⁶²Egwari et al., 1995) of the anaerobic microflora of the FGT found antibiotic treatment to significantly reduce the population and quantitative count of the anaerobic bacteria. This group consistently isolated anaerobic bacteria from both healthy volunteers and women with proven gynaecological infections, irrespective of antibiotic treatment. The main genera isolated were *Bacteroides*, *Clostridium*, *Peptostreptococcus*, *Porphyromonas* and *Prevotella* spp. Unlike in the Gambia study (¹⁴Demba et al., 2005), they did not isolate species previously found to be significantly associated with BV, namely *G. vaginalis*, *M. hominis*, *U. urealyticum* and *Mobiluncus* spp. *In vitro* studies have shown that exposure to antibiotics is a risk factor for loss of vaginal colonisation by hydrogen peroxide-producing lactobacilli (²⁷Wilks et al., 2004). Topical antibiotics are particularly effective at decimating vaginal lactobacilli (¹⁶³Larsson et al., 2004). In a more recent study (¹⁶⁴Anukam et al., 2006c) of *Lactobacillus* VMF of women of reproductive age, Nigeria, the prevalence of BV was 14.2%. However the intermediate Nugent score was much higher at 51%. This study found *L. iners* to be the most common lactobacilli. As in the Gambia study (¹⁴Demba et al., 2005), *M. hominis* was most common in BV cases.

Other studies have focused on the intravaginal practices of women. One study in Zimbabwe found users to be more likely to have disturbed VMF (¹⁰³van de Wijgert et al.,

2000). In this study the absence of lactobacilli from the VMF was associated with being positive for HIV virus. Recent sex was associated with increased BV prevalence while vaginal drying was associated with lower prevalence of BV. More recently in north-western Tanzania ¹⁶⁵Baisley *et al.* (2009) found high a prevalence of BV among HSV-2 positive female facility workers. Most studies on VMF have been carried out on high risk populations, particularly those involved in commercial sex work. One such prospective study of BV microflora and other risk factors for vulvovaginal candidiasis (VVC) among sex workers found the prevalence of BV to be 37.1% (⁵⁷McClelland *et al.*, 2009).

Lactobacillus colonization was associated with 4-fold increase in the likelihood of symptomatic VVC, and BV was associated with reduced risk of VVC. This finding is similar to the Malawi study of pregnant women (¹⁶⁰Taha *et al.*, 1999) which found the prevalence of BV to be lower among women with candidiasis. It has previously been shown that *Candida* infection favors low vaginal pH (⁹⁰Hillier *et al.*, 1992). Peak estrogen levels during the middle part of the cycle are thought to stimulate candidiasis (¹⁶⁶Mishell *et al.*, 1971). The symptoms of candidiasis have been observed to be more frequent during the latter part of the menstrual cycle (¹⁶⁷Spinillo *et al.*, 1995), whereas BV is prevalent in the first week of the menstrual cycle (¹²⁶Morison *et al.*, 2005).

A study of the prevalence of HSV-2 infection among Kenyan sex workers (¹⁰⁹Kaul *et al.*, 2007) found that altered VMF increases susceptibility of infection with *C. trachomatis* and *T. vaginalis*. In a similar population, a 10-year prospective study (¹⁰⁰McClelland *et al.*, 2006) found vaginal washing to increase the risk of acquiring HIV-1, findings similar to those of earlier studies (¹⁶⁰Taha *et al.*, 1999; ¹⁶⁸Taha *et al.*, 1998b; ¹⁰³van de Wijgert *et al.*, 2000). In high risk seronegative women in Kenya, a prospective study (¹⁰⁸Baeten *et al.*, 2009) found modifiable biological and behavioural factors to be associated with *Lactobacillus* colonisation. Vaginal washing and recent use of antibiotics decreased the presence of lactobacilli, findings similar to an earlier study in Nigeria (¹⁶²Egwari *et al.*, 1995). In this Kenyan cohort, an earlier report (¹¹Martin *et al.*, 1999) found colonisation by *Lactobacillus* to be associated with decreased incidence of HIV-1 and *N. gonorrhoeae*. Microbicidal properties of lactobacilli can be cidal to HIV-1 (⁵⁴Klebanoff and Coombs, 1991), while the acidic environment created by the lactic acid produced by

Lactobacilli inactivates the virus (¹¹Martin *et al.*, 1999). Further, ¹¹Martin *et al.* (1999) found a high prevalence rates of BV at screening and during follow-up respectively (36% and 33%) among African prostitutes, rates comparable to those found in a high risk population of women attending an STD clinic in Nairobi (¹³Bukusi *et al.*, 2006). ¹⁰⁸Baeten *et al.* (2009) also found the likelihood of *Lactobacillus* isolation to decrease with age, as was the case in an earlier study of Japanese women (¹²⁵Fujisawa *et al.*, 1992a). The finding of ¹⁰⁸Baeten *et al.* (2009) that a higher number of sexual partners was associated with detection of *Lactobacillus* however, is in contradiction with earlier studies (¹⁶⁹Beigi *et al.*, 2005; ¹⁷⁰Nagot *et al.*, 2007; ¹⁷¹Verstraelen *et al.*, 2010).

A study investigating the validity of the vaginal discharge diagnosis and treatment algorithm among pregnant and non-pregnant women in Nairobi, Kenya, found the rate of BV to be 9% (¹⁷²Fonck *et al.*, 2000). A more recent study within similar health service settings in Nairobi (¹³Bukusi *et al.*, 2006) found the prevalence of BV to be 44%, which implies that the former study may have underestimated its occurrence. The most common infections associated with vaginal discharge in the ¹⁷²Fonck *et al.* (2000) study were *Candida albicans* and *T. vaginalis*. This has been shown to be the case in other African studies (¹⁷³Alary *et al.*, 1998; ¹⁷⁴Mayaud *et al.*, 1998). The study identified the need to improve the existing algorithms for treatment of vaginal discharge in Kenya. A randomized trial in Mombasa, Kenya (¹⁷⁵McClelland *et al.*, 2008) showed that periodic treatment reduced the incidence of BV and promoted colonization with *Lactobacillus*. This would potentially lower the risk of acquisition of HIV-1 and other STIs (¹⁰⁸Baeten *et al.*, 2009; ¹⁰⁹Kaul *et al.*, 2007; ¹⁰⁰McClelland *et al.*, 2006). ¹³Bukusi *et al.*, 2006 found behavioural characteristics of the two genders to be independently associated with BV. The study identified lower socioeconomic status as a risk factor for BV. In a similar study on genital hygiene practices in Mombasa, Kenya, (¹⁷⁶Hassan *et al.*, 2007) found significant likelihood of BV among women who bathed less frequently. The latter study also identified lubrication with petroleum jelly or saliva to be risk factors for BV. It appears then that more risk factors may yet be identified as further research continues, but mainly among the much studied high risk populations.

From the foregoing discussion, it is apparent that in sampling for BV studies, bias may arise due to the use of centre-based recruitment common to almost all the studies so far. The only exception is the American population-based and Ugandan community-based studies. Recently (⁷⁶Smart *et al.*, 2004) sought to overcome this bias while studying risk factors for BV. They felt that existing studies had used small numbers of patients of highly selected or convenience samples, or poorly defined populations. In their methods, though with a fairly representative sample size, they ended up with a centre-based study at the Sydney Sexual Health Centre. It may be logical therefore to conclude that these studies may closely mirror the situation in the base populations, since BV is the most common gynaecologic complaint for which women seek medical attention (¹⁴³Spiegel, 2002; ⁶Nyirjesy, 2008). There is so far no known explanation for the observed racial differences in the prevalence of BV. Evidence has increasingly pointed to the intravaginal practices of African women. Insertion of cloths or detergents into a sensitive area like the vagina, where maintaining the balance requires the *status quo* physico-chemical properties be preserved, can be traumatic for the VMF. It is possible that these practices distort the balance, and further introduce microflora from the environment or other parts of the body. African women are 2.5 times more likely to douche than Caucasian women (¹⁷⁷Aral and Mosher, 1992).

The second pointer is that the VMF found in African women does not harbor the beneficial lactobacilli in large enough quantities. The benefits of a Lactobacillus-dominated microflora have already been discussed. A study in Nigeria (¹⁶⁴Anukam *et al.*, 2006c) found *L. iners* to be the most abundant lactobacilli. This species is known to be either a poor hydrogen peroxide producer or not at all (²⁶Vallor *et al.*, 2001). The hydrogen peroxide-producing lactobacilli are either absent or occur in significantly low quantities in African women (⁵⁷McClelland *et al.*, 2009), yet they protect against acquisition of BV (⁸⁹Eschenbach *et al.*, 1989; ⁵⁵Hawes *et al.*, 1996). It has also been observed that African women have higher vaginal pH compared to Caucasian women (¹⁷⁸Stevens-Simon *et al.*, 1994; ⁸⁵Royce *et al.*, 1999a), naturally predisposing them to acquisition of BV. Although (¹⁷⁹Fiscella and Klebanoff, 2004) concluded that it is unlikely this racial variation attributable to biological variation in vaginal pH, it should however be

noted that the microflora was defined only using Gram stain, which does not exhaustively distinguish the species of *Lactobacillus* present (⁹⁹De Backer *et al.*, 2007). These pointers urgently require further investigation given the disease burden of BV and the risk it poses to the acquisition of other STIs, several of which also affect the woman's sexual partner(s) and her yet-to-be-born baby.

2.3.2 Risk Factors and Mechanisms of Bacterial Vaginosis

The risk factors for BV are well documented, have been reported in health and disease, and many of them are to a certain extent mutually dependent. They include sexual behaviours such as multiple sex partners (¹⁸⁰Avonts *et al.*, 1990; ¹⁸¹Barbone *et al.*, 1990; ⁵⁵Hawes *et al.*, 1996; ⁷⁶Smart *et al.*, 2004; ¹³Bukusi *et al.*, 2006), female sex partners (¹⁸²Marrazzo, 2007; ¹⁸³Marrazzo *et al.*, 2010; ⁷⁶Smart *et al.*, 2004), gravidity (⁷⁶Smart *et al.*, 2004), frequency of vaginal intercourse (²⁶Vallor *et al.*, 2001), inconsistent condom use (²⁶Vallor *et al.*, 2001; ⁷⁷Hutchinson *et al.*, 2007; ¹⁸⁴Fethers *et al.*, 2008), douching (⁵⁵Hawes *et al.*, 1996; ¹⁵⁴Koumans *et al.*, 2007) and other intravaginal practices (¹⁰³van de Wijgert *et al.*, 2000; ¹⁷⁶Hassan *et al.*, 2007; ¹⁸⁵Baeten *et al.*, 2010). Some risk factors are socially oriented like is smoking (⁷⁶Smart *et al.*, 2004), use of an intrauterine contraceptive device (¹⁸⁰Avonts *et al.*, 1990; ⁷⁶Smart *et al.*, 2004), low socioeconomic status (¹⁸⁶Goldenberg *et al.*, 1996; ¹⁸⁷Steele *et al.*, 2004; ¹³Bukusi *et al.*, 2006; ¹⁵⁴Koumans *et al.*, 2007) and male partner characteristics (¹³Bukusi *et al.*, 2006), while others are physiologic such as concomitant infection with *T. vaginalis* (¹³Bukusi *et al.*, 2006; ¹⁰⁹Kaul *et al.*, 2007), a previous history of STIs (¹⁸⁴Fethers *et al.*, 2008), HIV infection (¹²Sewankambo *et al.*, 1997), absence of hydrogen peroxide-producing lactobacilli in the vagina (⁵⁵Hawes *et al.*, 1996; ⁸⁴Antonio *et al.*, 2005), recent use of antibiotics (²⁶Vallor *et al.*, 2001), menses (⁷Keane *et al.*, 1997; ⁵Wilson, 2005) and use of vaginal medication, spermicide use (⁵Wilson, 2005). Black ethnicity (¹⁸⁸Ness *et al.*, 2003; ¹⁵⁴Koumans *et al.*, 2007) and nutritional factors (⁷⁴Ahluwalia and Grandjean, 2007) have also been shown to be risk factors for BV.

On the other hand, use of hormonal contraceptive has emerged as a protective factor (⁷⁶Smart *et al.*, 2004). The final mechanistic pathway for BV is the depletion of the normal vaginal lactobacillus flora and overgrowth of other anaerobic species (¹⁰⁴Hay, 2005). It is, however, unclear which factor precedes the other. Whatever the mechanism, for disease to occur, endogenous or exogenous bacterial pathogens must attain replication dominance in the lower FGT. The common occurrence of *G. vaginalis* in BV cases led to its association with this condition, supported by its subsequent isolation from the chorioamnion of women in preterm labor (¹⁸⁹Hillier *et al.*, 1988). Recent studies (¹⁹⁰Harwich *et al.*, 2010; ¹⁹¹Patterson *et al.*, 2010; ¹⁹²Yeoman *et al.*, 2010; ¹⁹³Lopes dos Santos Santiago *et al.*, 2011) show that different genotypes of *G. vaginalis* employ various mechanisms to exert their virulence. These include formation of biofilm alongside the production of vaginolysin, prolidase, sialidase and β -galactosidase. The enzymes produced by *G. vaginalis*, jointly with those of other pathogens, digest the vaginal mucins (¹⁹⁴Roberton *et al.*, 2005), and their activity is upregulated in BV cases (¹⁹⁵Briselden *et al.*, 1992; ¹⁹⁶Cauci *et al.*, 1998; ¹⁹⁷Olmsted *et al.*, 2003; ¹⁹⁴Roberton *et al.*, 2005; ¹⁹⁸Cauci *et al.*, 2008).

Figure 5- Some of the known risk factors for bacterial vaginosis.

black race
low socio-economic status
psychosocial stress
abuse/partner violence
sexual behaviour-related characteristics
lesbian orientation
vaginal douching
intra-vaginal practices e.g. vaginal drying
smoking
presence of an IUD
nutritional factors

There is controversy over whether sexual transmission of BV occurs despite available evidence of correlation between sexual contact and transmission. Studies indicate heterosexual penetrative sex to be the highest risk for transmission of BV when backed by certain backgrounds such as multiple of lifetime partners and the recent acquisition of a new sex partner (¹⁹⁹Hart, 1993; ²⁰⁰Nilsson *et al.*, 1997; ²⁰¹Shoubnikova *et al.*, 1997; ¹³⁵Schwebke *et al.*, 1999; ²⁰²Hellberg *et al.*, 2000; ²⁰³Calzolari *et al.*, 2000). Other indicated sexual behaviour include frequency of intercourse, sex during menses, sex with an uncircumcised male partner and earlier age at sexual debut. The sex related factors affect the healthy acidic environment of the normal VMF. Intravaginal practices (¹⁰³van de Wijgert *et al.*, 2000; ¹⁷⁶Hassan *et al.*, 2007; ¹⁸⁵Baeten *et al.*, 2010),

inconsistent condom use (²⁶Vallor *et al.*, 2001; ⁷⁷Hutchinson *et al.*, 2007; ¹⁸⁴Fethers *et al.*, 2008) and having multiple sex partners (¹⁸⁰Avonts *et al.*, 1990; ¹⁸¹Barbone *et al.*, 1990; ⁵⁵Hawes *et al.*, 1996; ⁷⁶Smart *et al.*, 2004; ¹³Bukusi *et al.*, 2006) lead to depletion of lactobacilli, which in turn alters the vaginal ecosystem. The resulting conditions make it easier for BV-associated species to colonise the vagina. Implicating sexual intercourse, it is known that semen, being alkaline, (⁵¹Boskey *et al.*, 1999; ¹⁰⁴Hay, 2005), alters the vaginal ecosystem, enabling the organisms associated with BV to overcome the inhibition of low pH (¹¹⁰Klebanoff *et al.*, 1991). Exposure to this imbalance occurs more frequently in women who have more partners (⁷⁶Smart *et al.*, 2004) and in those who use condoms inconsistently (²⁶Vallor *et al.*, 2001). The observation that women who used condoms consistently were less likely to have BV, implies that condoms may be useful both to protect against BV and to reduce the rate of recurrence (²⁶Vallor *et al.*, 2001).

Frequent intercourse (²⁶Vallor *et al.*, 2001) is similarly considered a risk factor for the acquisition of BV, which implies that following coitus *Lactobacillus* microflora may require time to recuperate from the onslaught. It has been suggested that the temporary imbalance of VMF that ensues serves sperm survival and translocation, and the female partner needs to abstain in order to allow for recovery (²⁰⁴Leppäluoto, 2008). Sexual transmission of BV is supported by some of the early studies (¹⁴⁴Gardner and Dukes, 1955). Gardner and Dukes successfully induced BV by inoculation of volunteers with vaginal fluid from patients with BV and further isolated *G. vaginalis* from the urethra in 45 of 47 male partners of women with BV. ²¹⁰Criswell *et al.* (1969) used a concentration of pure *G. vaginalis* culture to achieve similar outcomes. Later ²⁰⁶Pheifer *et al.* (1978) detected *G. vaginalis* in 27 of 34 partners of BV patients. Further studies found male carriage of *G. vaginalis* at a rate of 11.4% in the UK (²⁰⁷Dawson *et al.*, 1982) and of 4.5% in Sweden (²⁰⁸Holst *et al.*, 1984) respectively. The rate of male carriage of *G. vaginalis* may be actually be higher; urethral sampling is not regarded the optimal approach to this nature of study. Significantly higher rates of *G. vaginalis* were isolated from preputial swabs (²⁰⁹Kinghorn *et al.*, 1982). The presence of *G. vaginalis* in semen is also documented (²¹⁰Chattopadhyay and Teli, 1984; ²¹¹Elsner and Hartmann, 1987;

²¹²Hillier *et al.*, 1990; ²¹³Ison and Easmon, 1985; ²¹⁴Kjaergaard *et al.*, 1997; ²¹⁵Lam *et al.*, 1988). This may point to a prostatic reservoir. One study had a recovery rate of *G. vaginalis* in semen at 38% among 58 men attending an infertility clinic (²¹³Ison and Easmon, 1985). Prior to the description of "*Haemophilus vaginalis*" as the causative agent of BV (¹⁴⁴Gardner and Dukes, 1955), *G. vaginalis* had been described as the cause of prostatitis (²¹⁶Leopold, 1953). Despite existing evidence for sexual transmission of BV, contradictions exist when compared to established patterns of such infections.

Reports indicate increased incidence of BV in women who have sex with women (WSW) (²¹⁷Berger *et al.*, 1995; ²¹⁸Skinner *et al.*, 1996; ²¹⁹McCaffrey *et al.*, 1999; ²²⁰Fethers *et al.*, 2000; ⁷⁶Smart *et al.*, 2004; ¹⁸²Marrazzo, 2007; ¹⁸³Marrazzo *et al.*, 2010). The pathogenesis of BV in WSW is not known, and ²¹⁹McCaffrey *et al.* (1999) failed to find any link between BV and specific sexual practice in lesbians. However, receptive cunnilingus may be linked to BV in this group (²²¹Morris *et al.*, 2001a), probably due to similarities between the anaerobic bacteria associated with gingivitis and those associated with BV (⁸⁴Antonio *et al.*, 2005). The study found simultaneous vaginal and rectal colonization by hydrogen peroxide-producing lactobacilli to be associated with a lower prevalence of BV, compared with vaginal colonization alone. These reports contradict the view that heterosexual penetrative contact is a necessary prerequisite to the acquisition of BV. It is also documented that BV in sexually unexposed adolescents and virginal women (²²²Bump and Buesching, 1988; ¹³⁸Yen *et al.*, 2003; ²²³Tabrizi *et al.*, 2006), but at much lower rates compared to sexually active reproductive age women. The finding of a good concordance in VMF characteristics, namely the of presence of hydrogen peroxide-producing lactobacilli and BV-associated organisms (²²⁴Marrazzo *et al.*, 2002) could indicate the existence of some mode of female-to-female transmission of BV.

Despite substantive evidence to support the sexual transmission of BV, there is no documentation of the benefit of partner treatment (²²⁵Colli *et al.*, 1997; ²²⁶Mengel *et al.*, 1989; ²²⁷Moi *et al.*, 1989; ²²⁸Swedberg *et al.*, 1985; ²²⁹Vejtorp *et al.*, 1988;

²³⁰Vutyavanich *et al.*, 1993) as happens in classical STIs. Further, some reports on condom use for prevention are also not conclusive (²⁰¹Shoubnikova *et al.*, 1997; ²⁰³Calzolari *et al.*, 2000; ²³¹Chiaffarino *et al.*, 2004; ⁷⁶Smart *et al.*, 2004; ²³²Schwebke and Desmond, 2005; ²³³Bradshaw *et al.*, 2005). Others studies indicate some beneficial effect of condom use with regard to acquisition of BV (⁵⁵Hawes *et al.*, 1996; ²³⁴Ahmed, 2001; ²³⁵Baeten *et al.*, 2001; ⁷⁷Hutchinson *et al.*, 2007). As such BV differs from other known STIs for lack of evidence in support of simple male-to-female transmission of a known infectious disease agent. This observation also contradicts the classical cause-and-effect pathway popularly known as Hill's causality criteria (²³⁶Hill, 1965). The view has emerged that BV should be referred to as a sexually enhanced disease (²³⁷Verstraelen, 2008). It is apparent that sexual contact sets the scene for anaerobic overgrowth of bacteria usually present in the vagina or rectum, rather than introduction of new pathogens. The key determinants of vaginal are the hydrogen peroxide - producing lactobacilli (⁸⁴Antonio *et al.*, 2005). The acquisition of BV, is strongly associated with a lack or loss of hydrogen peroxide-producing lactobacilli (⁵⁵Hawes *et al.*, 1996) and even the effect of frequent intercourse depend on whether these are lost (²⁶Vallor *et al.* 2001), further supporting the view of a sexually enhanced disease (²³⁷Verstraelen, 2008). As long as no etiologic agent for BV is identified, there shall be freedom of description with regard to the pattern of disease it follows.

Other sexual behaviours that pose a risk of acquisition of BV include the practice of douching (⁵⁵Hawes *et al.*, 1996; ¹⁵⁴Koumans *et al.*, 2007). Following menses, douching has emerged as a strong predictor of BV. It is still unclear however, whether the douching is causal or done in response to existing symptoms of BV (²³⁸Schwebke *et al.*, 2004; ¹⁰⁴Hay, 2005). This practice endangers survival of the much-needed hydrogen peroxide producing lactobacilli (²³⁹Ness *et al.*, 2002; ¹⁶⁹Beigi *et al.*, 2005), worsening the adverse effect of menses (²⁴⁰Brotman *et al.*, 2008b). The effect is comparable to that of sexual intercourse during menses (²⁴¹Ness *et al.*, 2004). Vaginal douching, which describes the practice of intravaginal cleansing with a liquid solution following menses and /or sexual activity (²⁴²Fonck *et al.*, 2001; ²⁴³Funkhouser *et al.*, 2002; ¹⁸⁸Ness *et al.*, 2003; ²⁴⁰Brotman *et al.*, 2008b), causes transient reduction in the total bacterial counts

in the vagina (²⁴⁴Onderdonk *et al.*, 1992; ²⁴⁵Sutton *et al.*, 2006; ¹⁷⁶Hassan *et al.*, 2007) and leads to increased risk of infection (²⁴⁶Cone *et al.*, 2006). Done mainly for hygiene purposes, the practice is common among African-American and sub-Saharan African populations (¹⁰³van de Wijgert *et al.*, 2000; ²⁴³Funkhouser *et al.*, 2002; ²⁴⁰Brotman *et al.*, 2008b). The *US 2001 National Health and Nutrition Examination Survey* found the population prevalence of douching in the United States to be 22.4%, with highest rate of 50.2% among non-Hispanic black women (²⁵⁰Sutton *et al.*, 2006). While epidemiologic evidence may suggest the practice of douching to predispose to acquisition of BV, support of the temporal relationship is missing. ⁷⁷Hutchinson *et al.* (2007) found that the practice may sufficiently disrupt already-imbalanced VMF to create BV. One study found that cessation of douching significantly reduced the risk of acquiring BV (¹³⁶Brotman *et al.*, 2008a). Menstrual flooding (²⁴⁷Wilson *et al.*, 2007) and the accompanying high pH, weaken the adherence of lactobacilli to the vaginal epithelium. These factors partly explain why pregnant (¹³⁹Hay *et al.*, 1994) and postmenopausal women (²⁴⁸Hillier and Lau, 1997) have very low rates of BV.

Risk factors for BV that bear a social orientation include cigarette smoking (⁷⁶Smart *et al.*, 2004). Smoking poses an independent non-sexual risk factor for BV, which is directly proportional to the number of cigarettes smoked (²⁴⁹Goldenberg and Das, 2000; ²⁵⁰Mardh, 2000; ²⁵¹Wilson *et al.*, 2002; ⁷⁶Smart *et al.*, 2004). Cigarette smoke contains various chemicals like nicotine, cotinine, and benzo[a]pyrene diol epoxide, detected in cervical mucus of smokers. These chemicals either directly alter the VMF or deplete the Langerhans cells in cervical epithelium leading to local immunosuppression (¹³⁵Schwebke *et al.*, 1999). The use intrauterine contraceptive devices also poses a risk for developing BV (¹⁸⁰Avonts *et al.*, 1990; ⁷⁶Smart *et al.*, 2004). ⁷⁶Smart *et al.* (2004) also found gravidity to be independently associated with BV as is having been pregnant (²⁵²Moi, 1990). Having a history of STIs is also a risk factor for BV (¹⁸⁴Fethers *et al.*, 2008), although BV may be consequence of the STI. The STIs alter the vaginal physiology, raising the vaginal pH and thus making the environment less conducive to the survival of lactobacilli, thus promoting the development of BV (²⁵³Wiesenfeld *et al.*, 2003).

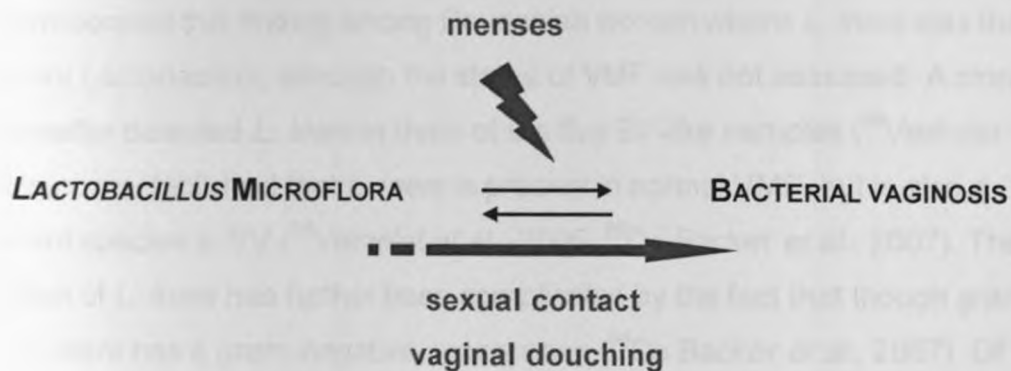


Figure 6- Basic elements of proposed mechanism for development of bacterial vaginosis

The absence of hydrogen peroxide-producing lactobacilli in the vagina predisposes to the acquisition of BV (⁵⁵Hawes *et al.*, 1996; ⁸⁴Antonio *et al.*, 2005). ²⁶Vallor *et al.* (2001) found persistent colonisation in 85% of women who initially harboured hydrogen peroxide-producing strains. Only 50% of those who lost colonization had these strains at the start of study. Even isolates of *L. crispatus* and *L. jensenii* that did not produce hydrogen peroxide were lost after the initial study visit. *Lactobacillus iners* was first described as a distinct species using 16S rRNA sequence divergence studies (²⁵⁴Falsen

et al., 1999). It is a facultative anaerobic micro-organism that does not grow on conventional *Lactobacillus* media like MRS or Rogosa agar, which explains why the species was not recognized earlier. It was subsequently described as part of the normal VMF in a Swedish study involving 23 women (⁹⁶Vasquez *et al.*, 2002). ⁹⁵Burton *et al.* (2003) corroborated this finding among Caucasian women where *L. iners* was the predominant *Lactobacillus*, although the status of VMF was not assessed. A cloning study thereafter detected *L. iners* in three of the five BV-like samples (³⁰Verhelst *et al.*, 2004). It is now established that *L. iners* is present in normal VMF, but is also a predominant species in BV (³¹Verhelst *et al.*, 2005; ⁹⁹De Backer *et al.*, 2007). The identification of *L. iners* has further been complicated by the fact that though gram-positive, *L. iners* has a gram-negative appearance (⁹⁹De Backer *et al.*, 2007). Of note is that *L. iners* is a very poor hydrogen peroxide producer (²⁶Vallor *et al.*, 2001).

In a recent study (²⁵⁵Ferris *et al.*, 2007) that analysed the VMF following treatment with topical metronidazole found *L. iners* to be predominant in all patients, except in one patient for whom treatment failed. Following the resolution of BV, *L. iners* is among the first *Lactobacillus* to recolonise the vagina. This in turn could render the patients more vulnerable to a new episode of BV due to the weak colonisation resistance of *L. iners*. Subsequently, in two women presenting with BV and harbouring nearly identical VMF, ²⁵⁶Fredricks *et al.* (2005) found that following treatment with oral metronidazole and resultant cure, relapse occurred in one of them. Thirty days post-treatment, the cured woman was found to be repopulated with *L. crispatus*, while the woman who relapsed harboured only *L. iners*. Although too small a sample size, these preliminary findings are in line with the prevailing hypothesis that recuperation of a stable *Lactobacillus* microflora is crucial to the long-term outcome of treatment of BV, and that the presence of abundant *L. iners* compromises this evolution. More studies are required to shed light on the role of *L. iners* in the normal VMF.

Black race (¹⁸⁸Ness *et al.*, 2003; ¹⁵⁴Koumans *et al.*, 2007) and nutritional factors (⁷⁴Ahluwalia and Grandjean, 2007) have also been shown to be risk factors for BV as has low social economic status (²⁵⁷Meis *et al.*, 1995; ¹⁸⁶Goldenberg *et al.*, 1996;

¹⁸⁷Steele *et al.*, 2004; ¹³Bukusi *et al.*, 2006; ¹⁵⁴Koumans *et al.*, 2007) and male partner characteristics (¹³Bukusi *et al.*, 2006). From the foregoing discussion it is apparent that women, and in particular those of black race, are naturally at the risk for developing BV, and that the risk is enhanced by factors that are more or less part of the normal life routine of the woman. This happens when the numbers of vaginal lactobacilli decline, allowing other anaerobes to overgrow. Alternatively, *Lactobacillus* loss may be secondary to the massive overgrowth of BV-associated bacteria. Little progress has been made in deciphering the exact sequence of pathologic events that result in the condition known as bacterial vaginosis.

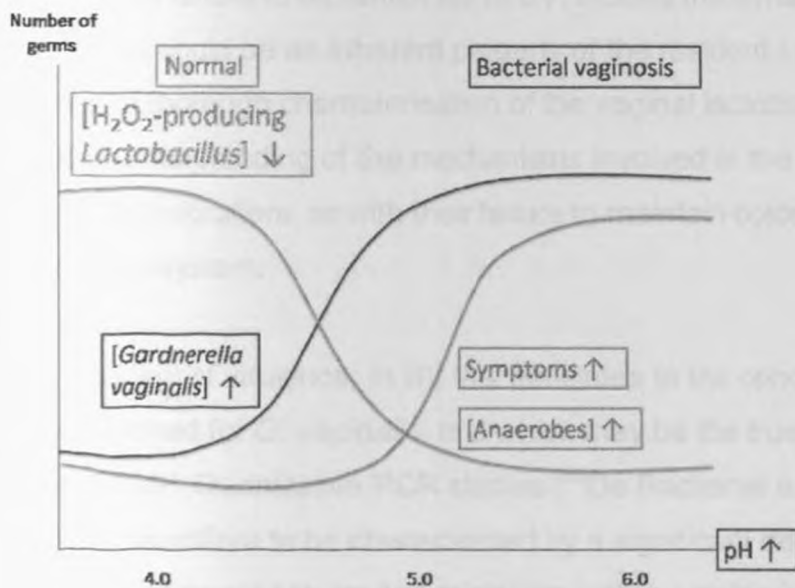


Figure 7-The interrelation between lactobacilli, vaginal pH, and bacterial overgrowth in the etiology of bacterial vaginosis.

2.3.3 Consequences and Pathology of Bacterial Vaginosis

In BV there occurs inverse relationship between numbers of hydrogen-producing lactobacilli and other anaerobic bacteria (⁸⁹Eschenbach *et al.*, 1989). This observation led researchers to determine which comes first; the loss of lactobacilli or anaerobic overgrowth. It is possible that under the influence of the menstrual cycle hormone fluctuations, the concentration of lactobacilli decrease thus giving a foothold to other anaerobes to overcome the inhibitions placed by the dominance of lactobacilli (⁷Keane *et al.*, 1997). In this study seven women volunteers who initially presented with normal VMF converted to intermediate microflora or BV during the early stages of the menstrual cycle. There was a decrease of lactobacilli within the first few days of menses, and in five of them the decrease in lactobacilli occurred on the day prior to a change in the status of VMF Gram stain. Consequently, the relatively depletion of lactobacilli, and the subsequent failure to replenish led to BV. Indeed the dynamism of the vaginal ecosystem could be an inherent property of the resident *Lactobacillus* (¹⁰⁴Hay, 2005). Studies of thorough characterisation of the vaginal lactobacilli in particular may provide a better understanding of the mechanisms involved in the stability of lactobacilli-dominated microflora, or with their failure to maintain colonisation dominance of the vaginal ecosystem.

As a concept of virulence, in BV this translates to the concentration of bacteria present, as established for *G. vaginalis*, and which may be the true determinant of an altered state of VMF. Quantitative PCR studies (⁹⁹De Backer *et al.*, 2007) recently showed grade III microflora to be characterised by a significant decline in concentrations of *L. crispatus*, compared to grade I microflora with a notable increase in the numbers of *L. iners*, *G. vaginalis* and *A. vaginae*. ²⁵⁸Menard *et al.* (2008) have obtained evidence that critical loads of *G. vaginalis* and *A. vaginae* are indeed predictive of the presence of BV. In BV, high numbers of *G. vaginalis*, *A. vaginae* and *L. iners* can be expected. The above findings challenge current diagnostics criterion defined by the critical loads of *G. vaginalis* and *A. vaginae*, implying that a significant proportion of BV goes undiagnosed by the current gold standard criteria for diagnosis, the Nugent score (⁷³Nugent *et al.*, 1991; ⁶Nyirjesy, 2008). The understanding of the pathogenesis and the therapeutic

resistance of BV may benefit from further studies on the role of these newly identified species, namely *L. iners* and *A. vaginae*. *Atopobium vaginae* was first described by ²⁵⁹Rodriguez-Jovita *et al.* (1999) as an isolate of the human vagina from an allegedly healthy Swedish woman. A fastidious gram-positive species, *A. vaginae* had never been cultured nor distinguished on Gram stain. Later ²⁹Geissdörfer *et al.* (2003) isolated *A. vaginae* from a tubo-ovarian abscess in a woman who presented with acute pelvic inflammatory disease. In a subsequent study by ²⁶⁰Burton *et al.* (2005), *A. vaginae* was identified as a prominent BV-associated species. This group reported the presence of *A. vaginae* in postmenopausal women with asymptomatic BV. Another group, ²⁶¹Ferris *et al.* (2004a) found *A. vaginae* in intermediate and BV microflora of a predominantly African American cohort. Other authors followed (³⁰Verhelst *et al.*, 2004; Verstraelen *et al.*, 2004) with the documentation of this organism in a cohort of pregnant and non-pregnant women with BV attending an outpatient clinic. In a separate incident, ²⁶²Hebb *et al.* (2004) independently reported the recovery of *A. vaginae* in ascending genital tract infection in women presenting with acute salpingitis in Kenya.

Now established as a predominant species in BV, *A. vaginae* has further been reported to be metronidazole-resistant (²⁹Geissdorfer *et al.*, 2003; ²⁶¹Ferris *et al.*, 2004a; ²⁶³Bradshaw *et al.*, 2006). Though the susceptibility of *A. vaginae* to metronidazole varies significantly among strains, (²⁶⁴De Backer *et al.*, 2006), the finding is important considering that metronidazole is the treatment of choice for BV. ²⁶³Bradshaw *et al.* (2006) found the rate of recurrence of BV to be strikingly higher in women concomitantly harbouring *A. vaginae* and *G. vaginalis*. ²⁵⁵Ferris *et al.* (2007) showed pre-treatment concentrations of *A. vaginae* to be highest in patients with completely or partially failed treatment of BV. More studies are needed to assess whether high pre-treatment concentrations of individual species, such as *A. vaginae*, are predictive of treatment outcomes for BV. The association of BV with serious sequelae such as chorioamnionitis, spontaneous abortion, preterm delivery, low birth weight, postpartum and post-abortion endometritis (²⁶⁵Gravett *et al.*, 1986; ²⁶⁶Hillier *et al.*, 1995; ²⁵⁷Meis *et al.*, 1995; ²⁶⁷Goldenberg *et al.*, 1997), and an increased susceptibility to HIV infection and other STIs (¹⁶¹Taha *et al.*, 1998a; ²⁵³Wiesenfeld *et al.*, 2003; ²⁶⁸Cherpes *et al.*, 2005), means

that current treatment outcomes result in unacceptably high recurrence rates (269 Koumans *et al.*, 2002; 263 Bradshaw *et al.*, 2006; 184 Fethers *et al.*, 2008).

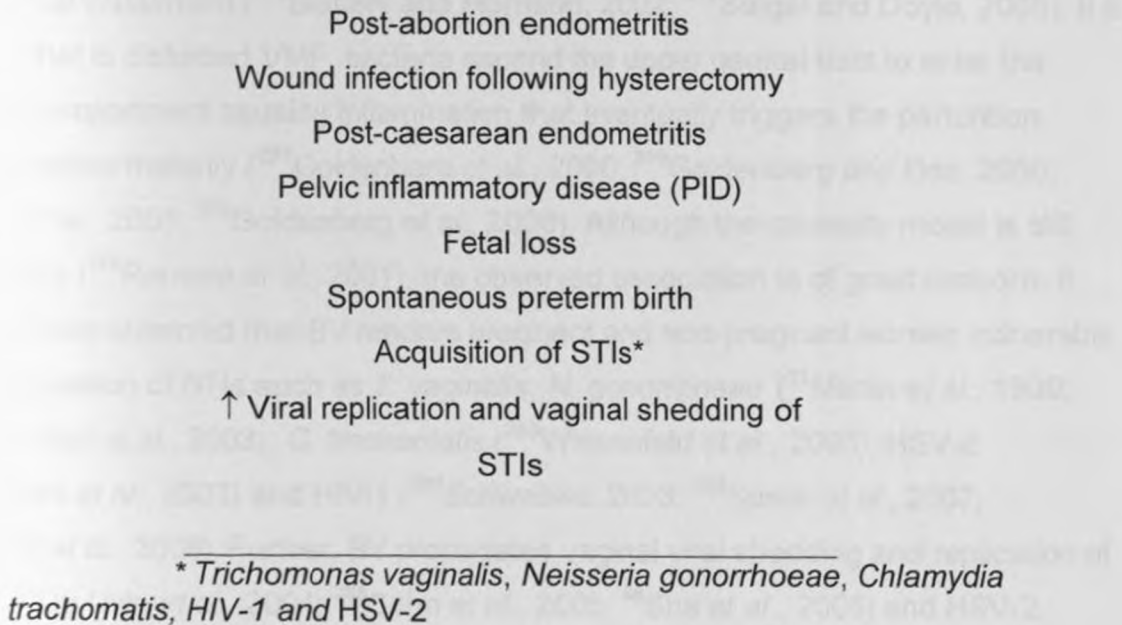


Figure 8- Known adverse consequences of bacterial vaginosis

Studies show that BV increases the risk of abdominal infection and cuff cellulitis following abdominal hysterectomy (²⁷⁰Persson *et al.*, 1996; ²⁷¹Soper *et al.*, 1990; ²⁷²Larsson *et al.*, 1991). Following caesarean section, infections involving ascending BV-associated micro-organisms could occur, a condition that also courses pelvic inflammatory disease (PID) (²⁷³Haggerty *et al.*, 2004). It is established that women who acquire a new sex partner and who show heavy growth of BV-associated microorganisms, are particularly at increased risk of PID (²⁷⁴Ness *et al.*, 2005). A major impetus to research in BV has been its consistent association with the occurrence of preterm birth, the leading cause of perinatal morbidity and mortality, as well as chronic neurological impairment (²⁷⁵Slattery and Morrison, 2002; ²⁷⁶Saigal and Doyle, 2008). It is believed that in disturbed VMF, bacteria ascend the upper genital tract to enter the amniotic compartment causing inflammation that eventually triggers the parturition cascade before maturity (²⁷⁷Goldenberg *et al.*, 2000; ²⁴⁹Goldenberg and Das, 2000; Romero *et al.*, 2001; ²⁷⁹Goldenberg *et al.*, 2008). Although the causality model is still under study (²⁷⁸Romero *et al.*, 2001), the observed association is of great concern. It has also been observed that BV renders pregnant and non-pregnant women vulnerable to the acquisition of STIs such as *T. vaginalis*, *N. gonorrhoeae* (¹¹Martin *et al.*, 1999; ²⁵³Wiesenfeld *et al.*, 2003), *C. trachomatis* (²⁵³Wiesenfeld *et al.*, 2003), HSV-2 (²⁸⁰Cherpes *et al.*, 2003) and HIV-1 (²⁸¹Schwebke, 2003; ²⁸²Spear *et al.*, 2007; ²⁸³Atashili *et al.*, 2008). Further, BV propagates vaginal viral shedding and replication of HIV-1 (²⁸⁴Cu-Uvin *et al.*, 2001; ²⁸⁵Cohn *et al.*, 2005; ⁵⁶Sha *et al.*, 2005) and HSV-2 (²⁶⁸Cherpes *et al.*, 2005), further enhancing their transmission.

It is estimated in endemic areas, nearly one-third of all new cases of HIV could be prevented if BV were cured (²⁸⁶Schwebke, 2005). Eradication of BV and accompanying mechanisms for restoring the VMF is now considered one of the most promising avenues to tackle the HIV burden (²⁸⁷Bolton *et al.*, 2008; ¹⁷⁵McClelland *et al.*, 2008). This is of particular importance in sub-Saharan African countries where the rates of HIV infection are sadly high. In Kenya about 1.42 million people were living with HIV infection in 2007, representing 7.1% of the population aged between 15 and 64 years. These figures are based on the Kenya Aids Indicator Survey (¹⁶KAIS, 2007), a

comprehensive household-based study conducted by the Kenya government and WHO during the last quarter of 2007. The study found prevalence among adults aged 15-49 years to have risen slightly from 6.7% in 2003 to the current 7.4%. Prevalence in rural Kenya rose significantly to 6.4% from the previous 5.3%, although urban prevalence rate remained higher at 8.4%. Throughout the country HIV infection rates varied widely from less than 1% among those living in the North Eastern province to about 15% in Nyanza province. Further, most infections occur in women (³⁴UNAIDS, 2004), yet 60% of the Kenyan adults over 18 years of age are women (¹⁷KNC, 1999).

2.3.4 Diagnosis of Bacterial Vaginosis

Bacterial vaginosis is a clinical syndrome which has been associated with a group of genital microorganisms and not a single etiologic agent as is the case in most infections. The development of BV follows transient stages between normal flora and definitive BV flora. A valid diagnostic tool for BV thus needs to accommodate this pattern, in addition to providing permanent record of the specimen. Stained smears of the bacterial morphotypes fulfill these requirements, making a reliable diagnosis subject to quality control (²⁸⁸Spiegel, 1991). Available laboratory tests are easy to standardize compared to the clinical criteria (⁹¹Hillier, 1993).

2.3.4.1 Clinical Diagnosis and Commercial Point of Care Tests

In the past the 'gold standard' for diagnosis of BV was the clinical criteria where at least three of the following signs had to be present (¹⁶³Amsel *et al.*, 1983):

- an adherent and homogenous grayish-white vaginal discharge
- vaginal pH exceeding 4.5
- the presence of so-called "clue cells" - vaginal epithelial cells coated with bacteria that obscure the peripheral borders
- production of fishy or amine odour on addition of a 10% potassium hydroxide solution (positive *whiff* or *sniff* test)

The Amsel diagnostic criteria, which have proved handy in clinical settings, continue to be in use to date. They are however subjective and could lead to missed diagnosis. On the other hand, a pH > 4.5 is considered the most sensitive criterion, whereas the presence of clue cells has been considered the single most specific predictor of BV (²⁸⁹Weir, 2004). However, (²⁹⁰Gutman *et al.*, 2005) believes that the use of any two of the Amsel's criteria is sufficient, and that a pH is ≥ 4.5 should be taken as positive diagnosis for BV. The accuracy of the Amsel's criteria is however likely to be primarily dependent on the experience of the evaluator.

In clinical practice several point-of-care tests are available commercially as follows:

- i) Osmetech Microbial Analyser™ (OMA™) is an electronic nose system in which the specimen vaginal swab is placed in a sealed vial. The sample is then passed over a polymer sensor of an array of volatile organic acids, where detection is based upon their molecular weight, structure and functional group (²⁹¹Hay *et al.*, 2003).
- ii) A self-test pH glove (CarePlan1 VpH, Selfcare, Unipath Diagnostics GmbH) can be used by women to measure vaginal pH regularly. This test is based on results of a study that demonstrate the importance of monitoring vaginal pH for the prevention of preterm birth (²⁹²Hoyme and Saling, 2004). The screening and treatment program enrolled 381 women who were instructed to contact their gynaecologist for further diagnosis and treatment at the observation of a pH ≥ 4.7 . The prematurity rate was 8% in the intervention group compared to 12% in the control. Premature rupture of membranes was registered in 22.8% versus 30.8% (P < 0.001) respectively.
- iii) The QuickVue Advanced pH & Amines test (Quidel Corporation), previously known as FemExam pH&Amines test, uses two plastic cards; the first to measure vaginal fluid pH and trimethylamine, and the second card to measure proline iminopeptidase activity of *G. vaginalis*. Positive reactions result in a qualitative colorimetric reaction (²⁹³West *et al.*, 2003).

iv) Osom BVBlue Test (Gryphus Diagnostics, L.L.C.) is a chromogenic office test based on the detection of elevated sialidase in vaginal fluid samples with mixed microflora (²⁹⁴Myziuk *et al.*, 2003).

v) The Affirm VPIII Microbial Identification Test (Becton Dickinson) detects *Candida spp.*, *G. vaginalis* and *T. vaginalis* in vaginal fluid. The test is based on nucleic acid hybridization and uses two distinct single-stranded probes for each organism, a capture probe and a color development probe. Results of the assay can be visualised (²⁹⁵Brown *et al.*, 2004).

2.3.4.2 Microscopy

The Gram stain is the method of choice for diagnosis of BV in research settings and is considered as the gold standard (²⁹⁶Spiegel *et al.*, 1983; ⁷³Nugent *et al.*, 1991; ²⁹⁷Mastrobattista *et al.*, 2000; ⁶Nyirjesy, 2008). For this test, high vaginal fluid collected on a glass slide is air-dried, fixed and stained and examined under oil immersion for the presence of specific bacterial morphotypes. This method has the advantage of being low cost, the availability of a permanent record of the specimen, is reproducible and slides are easy to transport and store (²⁹⁸Krohn *et al.*, 1989). In addition, Gram stains can be standardized by repeated evaluation by independent assessors, thus increasing diagnostic reliability. Unfortunately the test is not popular with medical practitioners as most are not familiar with its method, leaving most office diagnoses to the Amsel criteria (²⁹⁹McGregor *et al.*, 1998).

The Gram stain method has undergone some modification since its initial inception by ²⁹⁶Spiegel *et al.* (1983). According to these authors, BV is present if the *Lactobacillus* morphotypes are fewer than five per oil immersion field, and if there are more than five of the other vaginal morphotypes. If five or more lactobacilli and fewer than five other morphotypes are present per oil immersion field, the Gram stain is considered to be normal. The method was later modified to accommodate the observed spectrum of severity in altered VMF (⁷³Nugent *et al.*, 1991). These latter authors developed a score

card known as the Nugent scoring system. The score card takes into account three of the most commonly encountered bacterial morphotypes namely *Lactobacillus*, *Gardnerella* and *Bacteroides* and *Mobiluncus* spp. The abundance of each morphotype observed per oil immersion field is quantified on a range from 0 to 4+, and obtaining the sum to get the Nugent score. A Nugent score of 7 or higher is taken as positive diagnosis for BV. A score of 4 to 6 represents the intermediate VMF, while a score of 0 to 3 is considered as normal VMF.

Cell morphotype specific score	<i>Lactobacillus</i> morphotypes per field	<i>Gardnerella</i> & <i>Bacteroides</i> morphotypes per field	curved gram-variable rods per field
0	> 30	0	0
1	5 – 30	< 1	1-5
2	1 - 4	1 – 4	> 5
3	< 1	5 – 30	
4	0	> 30	

Figure 9- The Nugent Scoring criteria of bacterial cell morphotypes on a Gram-stained vaginal smear (Nugent et al., 1991).

An additional modification to the Gram stain includes two more categories, grade 0 to represent epithelial cells only and with no bacteria, and grade IV to cater for occurrence of epithelial cells covered with Gram-positive cocci (³⁰⁰Ison and Hay, 2002). The Ison and Hay criteria consider it normal for a woman who has recently used systemic or topical antibacterial agents to harbour no bacteria, whilst the presence of epithelial cells covered with Gram-positive cocci is significant. ³⁰¹Donders *et al.* (2002) defined as *aerobic vaginitis* a type of VMF that resembles the Hay-Ison grade IV and mainly includes group B-streptococci, *E. coli* and *Staphylococcus aureus*. The wet smear microbial profile of anaerobic vaginitis is characterized by intense shedding of the vaginal mucosa, leading to increase in the counts of immune cells, bare nuclei and vaginal pH. Decrease in lactobacilli and the simultaneous detection of gram-positive cocci and occurred in one third of the cases (³⁰²Sobel, 1994). Recently, ³¹Verhelst *et al.* (2005) have added yet another category of VMF termed grade-I like, which shows a characteristic pattern on Gram stain. The grade-I like VMF is strongly associated with spontaneous preterm birth (³⁰³Verstraelen *et al.*, 2007), while over 50% of VMF in this category are characterized by an overgrowth of *Bifidobacterium spp.* (³¹Verhelst *et al.*, 2005).

Figure 1.1: Gram stain of vaginal swab with grades as classified by ³⁰⁰Ison *et al.*, A-grade 0; B-grade I; C-grade II; D-grade III; E-grade IV.

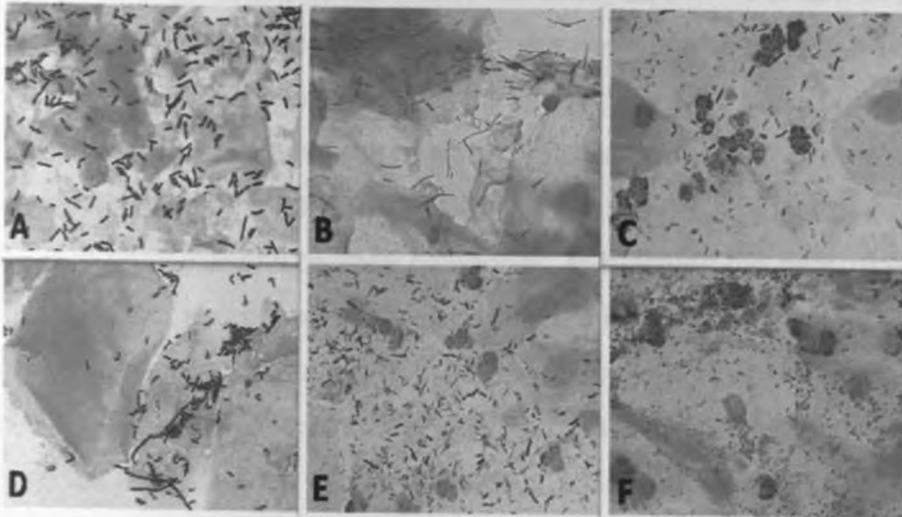


Figure 10- Gram stain of vaginal swabs with grades as classified by ³¹Verhelst *et al.*, (2005). A-grade 1a; B-grade 1b; C-grade 1ab; Dgrade1-like; E-grade II; F-grade III.

Although widely in use, the performance of the Nugent scoring system has raised concern in certain quarters. Firstly, the method lacks standardization of specimens.³⁰⁴Forsum *et al.* (2008) noted that variations in devices and procedures applied and differences in how the specimen is spread on the glass slide led to variations in homogeneity of the smear. Different fixation times and methods and variations in the choice of the area on the oil immersion field, all affect the interpretation of the Gram stain (³⁰⁵Forsum *et al.*, 2002; ¹⁶³Larsson *et al.*, 2004; ³⁰⁴Forsum *et al.*, 2008). Inter-observer reliability in scoring the Gram-stained vaginal smears has also been controversial, since there potential for diversity to arise based on the diagnostic method employed, since there is lack of meaningful categorisation of existing patterns of abnormal VMF (¹⁵⁷Tohill *et al.*, 2004). These authors suggest the inclusion of all the possible combinations of VMF categories, to highlight the heterogeneity inherent within existing Gram stain categories. In addition, it is documented that a significant proportion of Nugent's intermediate score is actually positive for BV based on Amsel's clinical criteria (³⁰⁶Taylor-Robinson *et al.*, 2003). ²⁵⁸Menard *et al.* (2008) reported that the presence of *A. vaginae* at a level of $\geq 10^8$ copies/mL and of *G. vaginalis* at a level of $\geq 10^9$ copies/mL is highly predictive of a positive diagnosis for BV. In this latter study, 57% of the intermediate score microflora was designated as BV. It is apparent that for Gram stain results to offer maximum benefit in the understanding of VMF, they need further delineation with molecular biology methods.

2.3.5 Treatment of Bacterial Vaginosis

Before 1980, BV was commonly regarded as a 'nuisance' infection, and remained mostly unrecognized by physicians. This scenario has since changed and BV is well recognized in association with a number of serious obstetrical and gynecological complications. Two antimicrobial agents, metronidazole and clindamycin, are the treatment of choice for BV as recommended by the Centers for Disease Control and Prevention (³⁰⁷CDC, 2006). Although standard treatment with metronidazole or clindamycin has a cure rate of 80-90%, BV recurs in 15-30% of the cases within three months, a condition known as recurrent bacterial vaginosis (³⁰⁸Cook *et al.*, 1992; ³⁰⁹Hay, 2000; ³¹⁰Wilson, 2004). There is currently no known optimal treatment for recurrent BV,

a situation that may be a reflection of the limitation in the understanding of the aetiology of BV compared with classical STIs. Treatment of BV primarily targets to alleviate the presenting symptoms since the pattern of the condition is poorly understood (³¹¹Joesoef and Schmid, 2005). It has been confirmed that in the absence of treatment, spontaneous remission of BV or episodic changes in the composition of VMF occurs (³¹²Brotman *et al.*, 2010). However, recurrence is quite frequent, typically in the period following menses. Even after successful therapy, the recurrence rates for BV are high and, long-term cure remains elusive to date (³⁰⁷CDC, 2006).

Grade II VMF responds poorly to clindamycin treatment, while grade III VMF readily resolves to the normal grade I (³¹³Rosenstein *et al.*, 2000; Taylor-Robinson *et al.*, 2003). Grade III VMF when treated with clindamycin gave better pregnancy outcomes than grade II, (³¹³Rosenstein *et al.*, 2000), which could be explained by the observed predominance of clindamycin-susceptible *L. iners* in grade III VMF (³⁰⁶Taylor-Robinson, 2003). Furhter, ⁹⁹De Baker *et al.* (2007) found the microscopic appearance of *L. iners* on a Gram stain to be atypical of *Lactobacillus* spp. Instead of strongly Gram positive rods, they appear as short Gram-negative coccobacilli. *L. iners* grows poorly on MRS agar, which explains why its abundant occurrence in grade III microflora has previously been overlooked on Gram stains of cultured specimens. The recent identification of a biofilm in BV (³¹⁴Swidsinski *et al.*, 2005) partly explains the metronidazole dilemma poor long-term cure rates with standard antibiotic treatment. ³¹⁵Swidsinski *et al.* (2008) observed the resurgence of a dense and active bacterial biofilm on the vaginal mucosa immediately after cessation of treatment. Mainly consisting of *G. vaginalis* and *A. vaginae*, is observed both in patients who reconverted to normal VMF and to intermediate state. In microbiologic terms, BV conforms to the defiant nature of conditions associated with biofilm formation. Indeed, biofilm formation is an adaptation directed toward concerted survival of the bacterial community through the avoidance of toxic/and therapeutic compounds (³¹⁶Miller and Bassler, 2001; ³¹⁷Camilli and Bassler, 2006). An *in vitro* model of *G. vaginalis* biofilm has recently been developed by ³¹⁸Patterson *et al.* (2007) that may be used in further therapeutic studies. It is however

worth noting that *in vivo* biofilm functions may be difficult to duplicate *in vitro*, given the accompanying physiologic factors *in vivo*.

Treatment regimens for BV have almost equal effectiveness irrespective of the route of administration (³¹⁹Livengood *et al.*, 1999; ³²⁰Sobel *et al.*, 2001). Short term response and cure rates following standard treatment are acceptable, apart from the recurrence or treatment failure in up to 29% of women at one month and in up to 70% by 3 months (³²¹Sobel *et al.*, 1993; ³²²Hillier *et al.*, 1993; ³²³Livengood *et al.*, 1994). Over time, recurrence rates can go up to 80% (³²⁴Eschenbach, 1993). It remains to be determined whether recurrence is due to reinfection or resistance to treatment. Combined treatment with metronidazole and azithromycin (³²⁵Schwebke and Desmond, 2007) and other 5-nitro-imidazoles (³²⁶Saracoglu *et al.*, 1998; ³²⁷Nunez and Gomez, 2005) have been explored, as well as vaginal acidifying gels (³²⁸Andersch *et al.*, 1986; ³²⁹van De Wijgert, 2001; ³³⁰Harwell *et al.*, 2003; ³³¹Brzezinski *et al.*, 2004). Other treatments include vaginal suppositories (³³²Petersen *et al.*, 2002; ³³³Wewalka *et al.*, 2002) and treated tampons (³³⁴Winceslaus and Calver, 1996; ³³⁵Cardone *et al.*, 2003). Probiotics currently in use include strains of *L. crispatus* (³³⁶Antonio and Hillier, 2003), while *L. rhamnosus* and *L. reuteri* (³³⁷Anukam *et al.*, 2006b; ¹⁶⁴Anukam *et al.*, 2006c) are under investigation for future application.

2.4 Microbial Analysis of the Vaginal Microflora

The use of culture techniques proved to be a powerful tool in the analysis of the diversity-rich VMF. However, because culture techniques have certain inherent limitations, its combined use with culture-independent PCR-based techniques has been recommended (¹²⁴Forsum *et al.*, 2005). Before the 21st Century, almost all research conducted on VMF depended on culture and phenotypic identifications. As may be apparent, the complex vaginal ecosystem could not possibly be exhaustively delineated by these methods alone, and errors occurred in identifications. The choice of culture medium and incubation conditions has inherent weakness leading to unnatural selection against certain organisms. In culture-based studies, storage and

transport of specimens from field/collection sites may also inadvertently compromise the quality.

2.4.1 Culture and Culture Methods

The use of culture is guided in general by use of selective or non-selective qualitative or quantitative analysis of either aerobic and/or anaerobic bacteria. For vaginal cultures, facultative anaerobic organisms are cultured using tryptic soy agar with 5% sheep blood, MacConkey's agar and mannitol-salt agar. For recovery of obligate anaerobes BMB (Brucella base blood agar with 5% sheep blood, hemin and menadione) is frequently used. Fastidious organisms are often isolated on chocolate agar. Although there may be no specific targeting, culture media do inherently select against some bacteria in the VMF, given that it is not possible to replicate all the factors that favour growth *in vivo*. *L. iners* failed to be detected in vaginal specimens before and after culture for such reasons. This organism does not grow on MRS agar that is in common use for isolation of lactobacilli. On the other hand, it shows up on Gram stain as Gram-negative coccobacillar rather than the typical Gram-positive bacillar appearance of lactobacilli (⁹⁹De Backer *et al.*, 2007). The difficulty of being unable to exactly replicate *in vivo* physiologic conditions often results in undesired selection. Selective media have been developed although they are not absolute and may be poisonous to certain strains within the genus.

Quantification of bacteria based on colony morphology, microscopy and physiologic properties forms the basis of phenotypic identification of cultured micro-organisms. In most cases these preliminary tests suffice. However in practice, clinical specimens are subjected to qualifying biochemistry and serology tests in order to draw conclusions. Relying entirely on phenotypic methods could result in error as morphology and microscopy may be influenced by subjective judgement and is therefore not sufficient for taxonomic assignment. In addition, biochemical identification tends to be labour-intensive and thus not suited for extensive studies (Forsum *et al.*, 2005). The VMF by its very nature is complex and variable, and to be able to discriminate all the genera and

species requires the application of an accurate methodology. For bacteria genera this is achieved by use of selective and semi-selective culture media. *Lactobacillus*, being the most abundant in a normal VMF can be isolated using both non-selective and selective media, mainly Rogosa and MRS agar (³³⁸Eschenbach *et al.*, 1988; ³³⁹Rogosa *et al.*, 1951). These media contain a high level of acetate and are of low pH, conditions that suit the growth of lactobacilli while suppressing many other strains. Other lactic acid-producing bacteria also flourish in these acidic conditions (³⁴⁰Jackson *et al.*, 2002). However, the same media selected against *L. iners*, a *Lactobacillus* species unable to grow on them even though it is present in over 60% of women (¹⁰²Anukam *et al.*, 2005; ²⁵⁶Fredricks *et al.*, 2005). The limitation of culture-based methods for the characterization of complex microflora has been described as 'the great plate count anomaly' (³⁴¹Staley and Konopka, 1985), since only a small fraction of the microorganisms present in a specimen thrive in culture.

2.4.1.1 Identification of cultured organisms

Some PCR-based methods for the identification of cultured *Lactobacillus* species have also been in use. Sequencing of the V1-V3 region of the 16S rRNA gene helped in identification of the organisms of the *L. acidophilus* group (³⁴²Kullen *et al.*, 2000; ³⁴³Tarnberg *et al.*, 2002) and subsequently randomly amplified polymorphic DNA analysis (RAPD) and amplified fragment length polymorphism (AFLP) techniques helped to differentiate between all species within this group (³⁴⁴Gancheva *et al.*, 1999). Two other molecular genetic methods, namely temporal temperature gradient electrophoresis (TTGE) and multiplex-PCR, were recently applied to distinguish between vaginal lactobacilli (⁹⁶Vasquez *et al.*, 2002). The amplification of the V2-V3 region of the 16S rDNA, followed by denaturing gradient gel electrophoresis (DGGE) to separate the different fragments, is also used (³⁴⁵Temmerman *et al.*, 2003; ³⁴⁶Walter *et al.*, 2000). Another technique that is in use for analysis and identification of *Lactobacillus* species in the VMF is tRNA-intergenic spacer length polymorphism PCR (tDNA-PCR). This method allows for rapid, discriminative and low cost identification of most cultivable bacteria except *Mycobacterium* spp., *Corynebacterium* spp. and *Neisseria* spp. (³⁴⁷Welsh and McClelland, 1991a; ³⁴⁸Vaneechoutte *et al.*, 1998). Using

universal primers for tDNA-PCR, the resulting PCR products can be separated by capillary electrophoresis, leading to unambiguous identification of most vaginal lactobacilli (³⁴⁹Baele *et al.*, 2002).

2.4.1.2 Principle of tDNA-PCR

The principle of tDNA-PCR relies on the amplification of the intergenic spacers between tRNA genes by use of consensus primers, which are complementary to the highly conserved edges of the flanking tRNA genes and are directed outwardly (Figure 11). The resulting PCR fragments can be separated by capillary electrophoresis, generating a species specific fingerprint, which can then compared to an existing database library for identification.

³⁴⁹Baele *et al.* (2002) applied tDNA-PCR using universal primers, followed by separation of the PCR products by capillary electrophoresis. To determine the discriminatory power of this technique, it was tested within the genus *Lactobacillus* and they were able to discriminate 21 of 37 tested species. It has also been applied to other species such as *Acinetobacter* (³⁵⁰Ehrenstein *et al.*, 1996; ³⁵¹Wiedmann-al-Ahmad *et al.*, 1994), *Enterococcus* (³⁵²Baele *et al.*, 2000), *Legionella* (³⁵³De Gheldre *et al.*, 2001), *Listeria* species (³⁴⁸Vaneechoutte *et al.*, 1998), *Staphylococcus* (³⁵⁴De Gheldre *et al.*, 1999; ³⁵⁵Maes *et al.*, 1997) and *Streptococcus* (³⁵⁴De Gheldre *et al.*, 1999). ³⁰Verhelst *et al.* (2004) combined culture with tDNA-PCR (³⁵⁶Welsh and McClelland, 1991b; ³⁵²Baele *et al.*, 2000; ³⁴⁹Baele *et al.*, 2002) and sequencing of the 16S rRNA-gene to characterize the VMF. tDNA-PCR is one of the few approaches that enable differentiation of most *Lactobacillus* species, including *L. crispatus*, *L. gasseri* and *L. iners*, which were previously all described as the *L. acidophilus* complex. This technique allows rapid, discriminative and low cost identification of most cultivable bacteria, although the primers currently used do have some limitations in differentiating certain species (³⁵⁶Welsh and McClelland, 1991b).

2.4.1.3 Capillary Electrophoresis

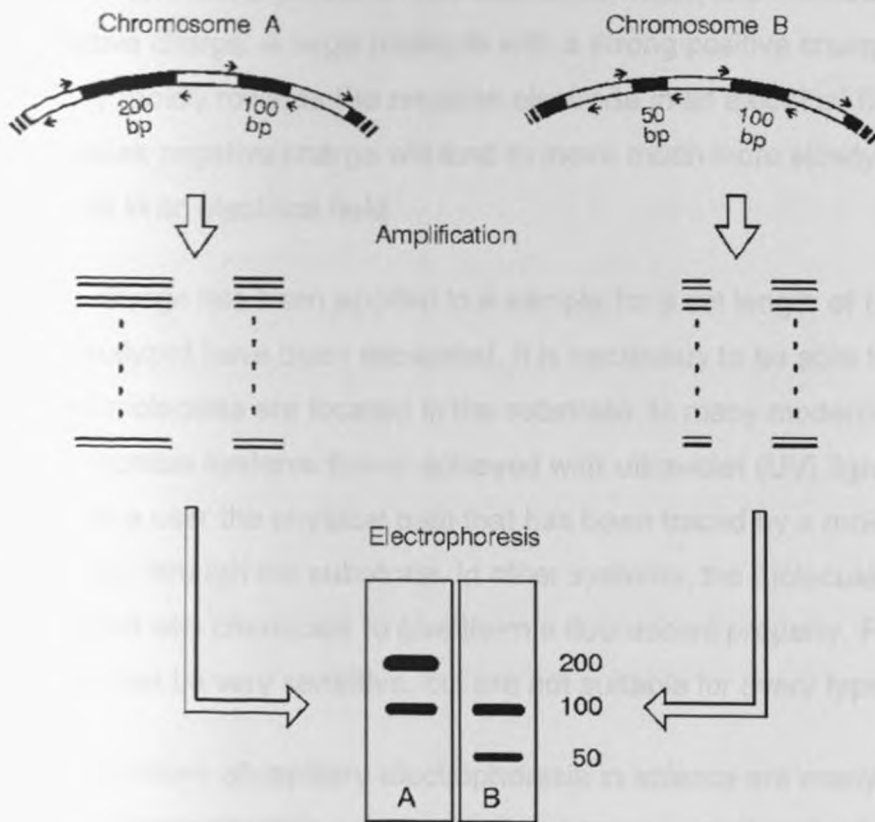
Capillary electrophoresis is a technique that is used to separate out molecules by their size and electrical charge. Analysis relies on the principle that molecules possess differing electrical charges and differing weights. Thus, when molecules in a substrate are exposed to an electrical field, different molecules move in different directions and at differing speeds within the substrate.

When a substrate has an electrical field applied to it by means of one electrode at either end of the field, positively charged molecules will move towards the negative electrode, and negatively charged molecules will move towards the positive electrode.



The principle of DNA interrogative polymerization length analysis (DIPAL)

INTERREPEAT SPACER LENGTH POLYMORPHISM ANALYSIS



- Consensus primers, outwardly directed
- Repeat element
- Interrepeat spacer

Figure 11: The principle of tRNA intergenic polymorphism length analysis (tDNA-PCR).

The relative speed at which the molecule will move through the substrate is determined by a characteristic known as the hydrodynamic size of the molecule. The hydrodynamic size of a molecule depends on two factors, its mass, and the strength of its positive or negative charge. A large molecule with a strong positive charge will tend to move relatively rapidly towards the negative electrode in an electrical field. A small molecule with a weak negative charge will tend to move much more slowly towards the positive electrode in an electrical field.

Once a charge has been applied to a sample for a set length of time, and the molecules being analyzed have been separated, it is necessary to be able to visualize where the different molecules are located in the substrate. In many modern capillary electrophoresis systems this is achieved with ultraviolet (UV) light. The UV illumination can show a user the physical path that has been traced by a molecule, or set of molecules, through the substrate. In other systems, the molecules to be analyzed are first treated with chemicals to give them a fluorescent property. Fluorescent detection methods can be very sensitive, but are not suitable for every type of molecule.

The applications of capillary electrophoresis in science are many and varied.

Capillary electrophoresis sequencing is a process, used particularly by geneticists, in which capillary electrophoresis is used to analyze DNA. Food scientists may use capillary electrophoresis protein analysis to determine the different protein content characteristics of food. In medical research, capillary electrophoresis has many applications, such as the analysis of antibodies, and the study of how they bind to other molecules.

The instrumentation needed to perform capillary electrophoresis is relatively simple. The system's main components are a sample vial, source and destination vials, a capillary, electrodes, a high-voltage power supply, a detector and a data output and handling device. The source vial, destination vial and capillary are filled with an electrolyte such as an aqueous buffer solution. To introduce the sample, the capillary inlet is placed into a vial containing the sample and then returned to the source vial

(sample is introduced into the capillary via capillary action, pressure, or siphoning). The migration of the analytes is then initiated by an electric field that is applied between the source and destination vials and is supplied to the electrodes by the high-voltage power supply. It is important to note that all ions, positive or negative, are pulled through the capillary in the same direction by electroosmotic flow. The analytes separate as they migrate due to their electrophoretic mobility and are detected near the outlet end of the capillary. The output of the detector is sent to a data output and handling device such as an integrator or computer. The data is then displayed as an electropherogram, which reports detector response as a function of time. Separated chemical compounds appear as peaks with different migration times in an electropherogram.

Separation by capillary electrophoresis can be detected by several detection devices. The majority of commercial systems use UV or UV-Vis absorbances their primary mode of detection. In these systems, a section of the capillary itself is used as the detection cell. The use of on-tube detection enables detection of separated analytes with no loss of resolution. In general, capillaries used in capillary electrophoresis are coated with a polymer for increased stability. The portion of the capillary used for UV detection, however, must be optically transparent. Bare capillaries can break relatively easily and, as a result, capillaries with transparent coatings are available to increase the stability of the cell window. The path length of the detection cell in capillary electrophoresis (~ 50 micrometers) is far less than that of a traditional UV cell (~ 1 cm). According to the Beer-Lambert law, the sensitivity of the detector is proportional to the path length of the cell. To improve the sensitivity, the path length can be increased, though this results in a loss of resolution. The capillary tube itself can be expanded at the detection point, creating a "bubble cell" with a longer path length or additional tubing can be added at the detection point. Both of these methods, however, will decrease the resolution of the separation (³⁵⁷Skoog *et al.*, 2007b).

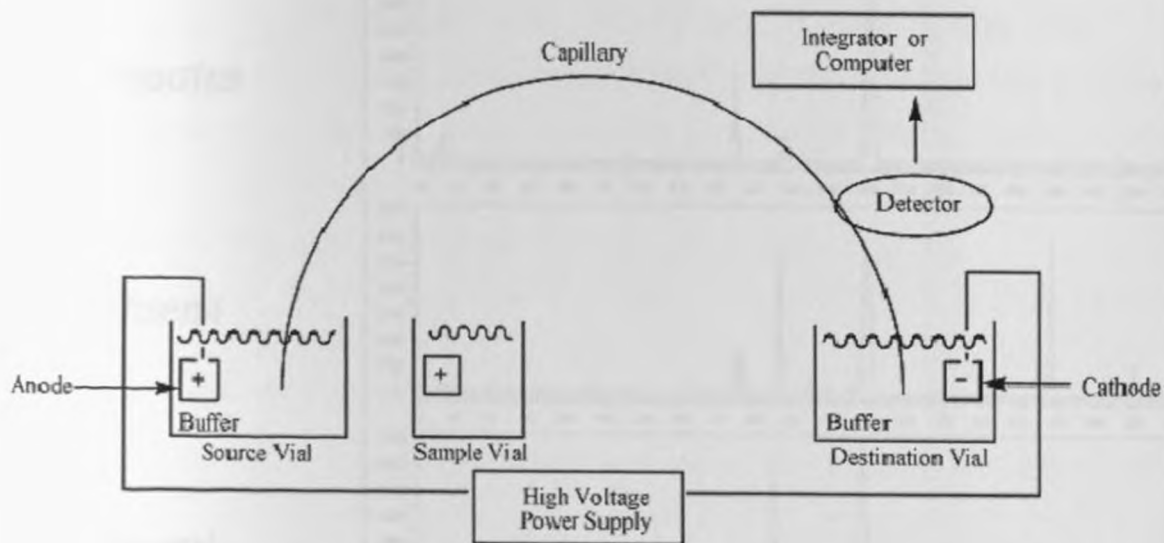
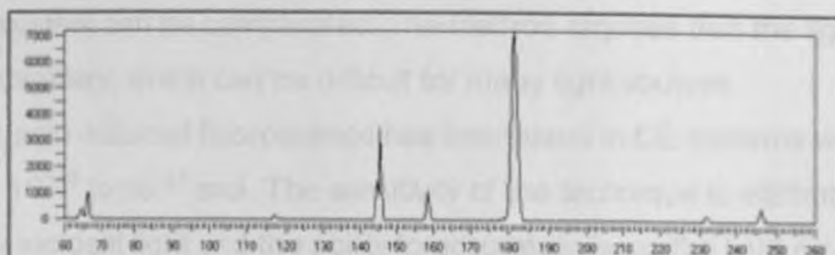
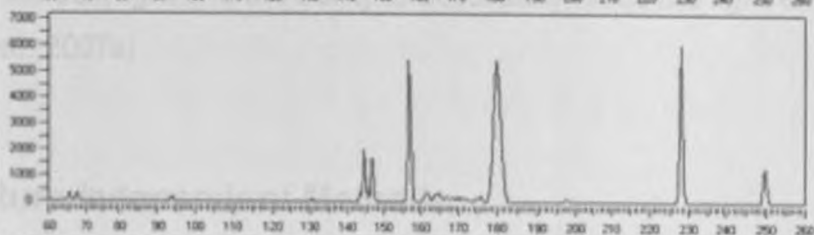


Figure 12 shows a basic schematic of a capillary electrophoresis system.

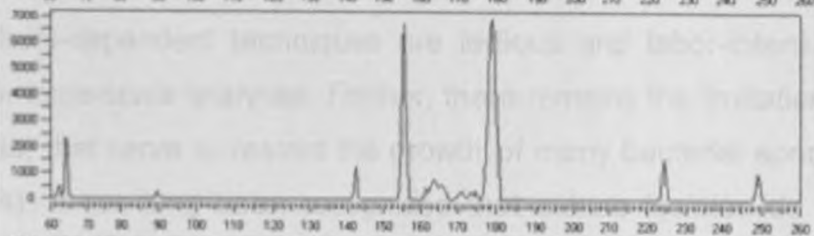
L. crispatus



L. jensenii



L. gasseri



L. iners

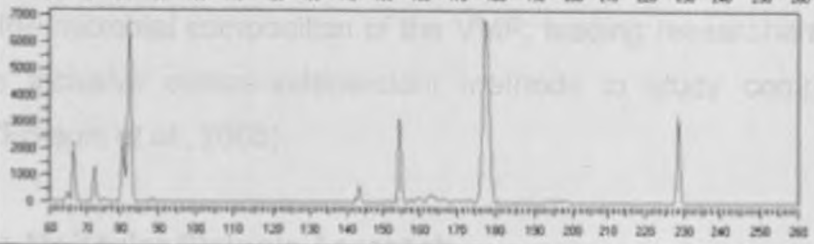


Figure 13- Illustration of different *Lactobacillus* tDNA patterns obtained in a capillary electrophoresis system.

Fluorescence detection can also be used in capillary electrophoresis for samples that naturally fluoresce or are chemically modified to contain fluorescent tags. This mode of detection offers high sensitivity and improved selectivity for these samples, but cannot be utilized for samples that do not fluoresce. The set-up for fluorescence detection in a capillary electrophoresis system can be complicated. The method requires that the light beam be focused on the capillary, which can be difficult for many light sources (357 Skoog *et al.*, 2007b). Laser-induced fluorescence has been used in CE systems with detection limits as low as 10^{-18} to 10^{-21} mol. The sensitivity of the technique is attributed to the high intensity of the incident light and the ability to accurately focus the light on the capillary (358 Skoog *et al.*, 2007a).

2.4.2 DNA-based Culture-independent Methods

As mentioned earlier, culture-dependent techniques are tedious and labor-intensive, making them unsuitable for large-scale analyses. Further, there remains the limitation of reliance on selective media, that serve to restrict the growth of many bacterial species (32 Verstraelen *et al.*, 2004). It has long been recognized that culture isolates do not reflect the true picture of the microbial composition of the VMF, leading researchers to look for alternative more inclusive culture-independent methods to study complex microbial communities (124 Forsum *et al.*, 2005).

2.4.2.1 Advantages of the Molecular Biologic Approach

PCR-based methods can be performed directly on vaginal samples without cultivation. As a result, species that would have been omitted due to set culture conditions such as availability of substrates, temperature, carbon dioxide, oxygen and pH are of necessity detected. The issues that arise with transport and preservation of specimens are also subverted by the use of PCR methods since only the intact nucleic acids are required. The 16S rRNA genes are universal in all bacteria, making them an ideal target for amplification and subsequent detection. These genes possess regions of sequence similarity that form the templates to be used with broad-range primers. Such primers amplify the 16S rDNA of almost all known bacterial species. Another advantage of using

the universal 16S rRNA bacterial genes is that variable regions with differing sequences also exist between the broad-range PCR priming sites. This sequence polymorphism of the variable regions allows for species differentiation, although there are certain exceptions as in the case of *Escherichia coli* and *Shigella* species, due to identical 16S rDNA sequences. Determination of the 16S rDNA sequences in a sample allows one to delineate members present in the community. There is a further advantage in the use of 16S rRNA gene. The more dissimilar sequences are, the greater their evolutionary distance, which makes the 16S rRNA gene useful for establishing phylogenetic relationships of bacteria species in cultured and uncultured specimens. The use of 16S rRNA gene culture-independent methods has resulted in an amazing added diversity and previously unknown bacteria phyla have been recognized and added to the GenBank (³⁵⁹Woese, 1987; ³⁶⁰Rappe and Giovannoni, 2003).

2.4.2.2 PCR: The Polymerase Chain Reaction

2.4.2.2.1 PCR: Introduction

PCR is a technique used to amplify the number of copies of a specific region of DNA in order to produce enough DNA to be adequately tested. PCR has become one of the most widely used techniques in molecular biology. It is a rapid and simple means of producing relatively large numbers of copies of DNA molecules from minute quantities of source DNA material.

2.4.2.2.2 The principle of PCR

The purpose of PCR is to make a huge number of copies of a gene. This is necessary to have enough starting template for further analysis.

2.4.2.2.3 The basic steps of PCR

There are three basic steps in a PCR, which are usually repeated for 30 or 40 cycles. This is done on an automated cycler, which can heat and cool the tubes with the reaction mixture in a very short time.

The basic PCR steps are:

1. Denaturation (at 94 °C). During this step, the double strand melts open to single stranded DNA, and all enzymatic reactions stop.
2. Hybridization or annealing (at 54 °C). At this step, the single-stranded sample DNA is "mixed" with a probe, and under the right conditions, hydrogen bonds form between this probe and its complementary sequence in the sample DNA. The double-helix structure is re-created.
3. DNA synthesis or replication (at 72 °C). This step presents the ideal temperature for the polymerase to act. Here, the primer (a piece of DNA that is base paired to the template strand in such a way that the 3' end of it is available to serve as the starting point for the new DNA) starts the process of "multiplying" the amount of DNA by attracting bases to attach to it. Primers that are on positions with no exact match get loose again and do not give an extension of the fragment.

The PCR was first performed in 1983 by a scientist at the Cetus Corporation in California known as Kary Mullis. He worked on the idea to replicate DNA in the lab for two years, and in 1985 published and filed a patent for his idea (³⁶¹Mullis et al., 1086). In 1993 he won the Nobel Prize in Chemistry for his work. The PCR procedure takes advantage of DNA polymerase enzymes and synthetic oligonucleotides to make many copies of a specific fragment of DNA. And because the reaction mixture contains primers complementary to both strands of DNA, the products of the DNA synthesis can themselves be copied with the opposite primer. The PCR utilizes:

- **Template DNA** - the starting DNA of interest.
- **Two Primers** - short, single-stranded, synthesized pieces of DNA that complement sequences on each side of the region of the template DNA that is being amplified.
- **Thermostable DNA Polymerase** - typically *Taq* (*Thermus aquaticus*), a heat stable enzyme capable of adding nucleotides to a growing DNA strand.
- **dNTPs** - a supply of the 4 nucleotides needed to make the new DNA strands.

- **Cationic Magnesium** - a cofactor for the polymerase.
- **Appropriately Buffered Solution** - to maintain the pH and salt concentrations appropriate for the polymerase

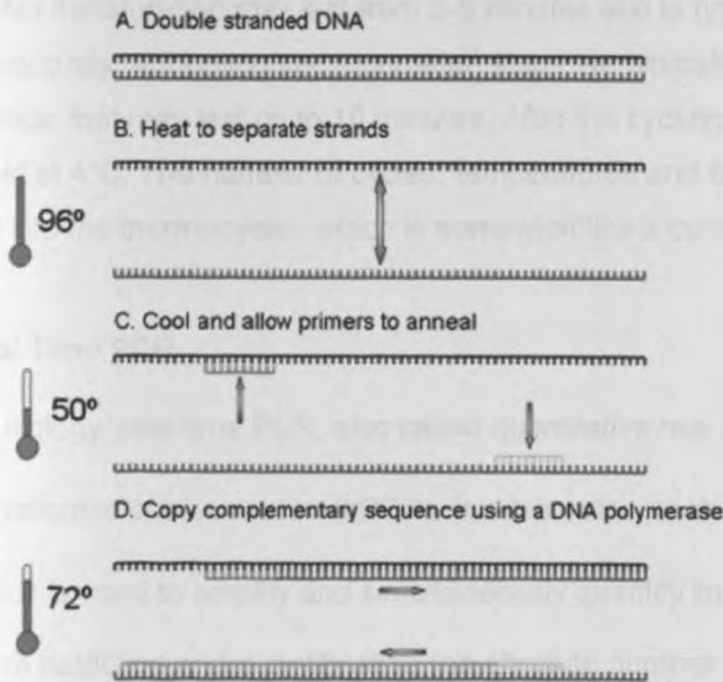


Figure 14- Illustration of the steps in PCR

Once these components are combined they go through a series of temperature changes (cycles), repeatedly, in a machine called a thermocycler. This process will generate copies of the DNA segment of interest exponentially. In other words, if one starts with 2 copies of the DNA segment of interest, after 20 cycles they will theoretically have $2^{(nth)} = 2^{(20th)} = 1,000,000$ copies of that segment. Each cycle consists of three parts: denaturation (D), annealing (A), and elongation or extension (E). Denaturation separates the double-strands of the DNA molecule at a relatively high temperature of 90-96°C, annealing allows the primer sequences to match and bind to the flanking regions of the target area at a moderate temperature between 40-70°C, and elongation or extension occurs as the polymerase adds nucleotides to the growing strand at 68-72°C. The initial denaturation may last from 2-5 minutes and is typically followed by cycles of 30 seconds. Annealing and elongation steps are typically 1 minute each, with a final elongation that may last up to 10 minutes. After the cycling is complete, the PCR product is held at 4°C. The number of cycles, temperatures and time lengths are programmed into the thermocycler, which is somewhat like a computerized heat block.

2.4.2.2.4 Real Time PCR

In molecular biology, real-time PCR, also called *quantitative real time PCR* (qPCR) or *kinetic polymerase chain reaction* (kPCR), is a laboratory technique based on the PCR, which is used to amplify and simultaneously quantify targeted DNA molecule. It enables both detection and quantification (as absolute number of copies or relative amount when normalized to DNA input) of one or more specific sequences in a DNA sample. The procedure follows the general principle of PCR; its key feature is that the amplified DNA is detected as the reaction progresses in *real time*, a new approach compared to standard PCR, where the product of the reaction is detected at its end. Two common methods for detection of products in real-time PCR are:

- (1) non-specific fluorescent dyes that intercalate with any double-stranded DNA,
and

(2) sequence-specific DNA probes consisting of oligonucleotides that are labeled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary DNA target.

(1) Real-time PCR with double-stranded DNA-binding dyes as reporters:

A DNA-binding dye binds to all double-stranded (ds)DNA in PCR, causing fluorescence of the dye. An increase in DNA product during PCR therefore leads to an increase in fluorescence intensity and is measured at each cycle, thus allowing DNA concentrations to be quantified. However, ds-DNA dyes such as SYBR Green will bind to all ds-DNA PCR products, including nonspecific PCR products such as primer dimers. This can potentially interfere with or prevent accurate quantification of the intended target sequence.

1. The reaction is prepared as usual, with the addition of fluorescent ds-DNA dye.
2. The reaction is run in a Real-time PCR instrument, and after each cycle, the levels of fluorescence are measured with a detector; the dye only fluoresces when bound to the ds-DNA (i.e. the PCR product). With reference to a standard dilution, the ds-DNA concentration in the PCR can be determined.

(2) Fluorescent reporter probes detect only the DNA containing the probe sequence; therefore, use of the reporter probe significantly increases specificity, and enables quantification even in the presence of non-specific DNA amplification. Fluorescent probes can be used in multiplex assays—for detection of several genes in the same reaction—based on specific probes with different-coloured labels, provided that all targeted genes are amplified with similar efficiency. The specificity of fluorescent reporter probes also prevents interference of measurements caused by primer dimers, which are undesirable potential by-products in PCR. However, fluorescent reporter probes do not prevent the inhibitory effect of the primer dimers, which may depress accumulation of the desired products in the reaction.

The method relies on a DNA-based probe with a fluorescent reporter at one end and a quencher of fluorescence at the opposite end of the probe. The close proximity of the reporter to the quencher prevents detection of its fluorescence; breakdown of the probe by the 5' to 3' exonuclease activity of the Taq polymerase breaks the reporter-quencher proximity and thus allows unquenched emission of fluorescence, which can be detected after excitation with a laser. An increase in the product targeted by the reporter probe at each PCR cycle therefore causes a proportional increase in fluorescence due to the breakdown of the probe and release of the reporter.

1. The PCR is prepared as usual, and the reporter probe is added.
2. As the reaction commences, during the annealing stage of the PCR both probe and primers anneal to the DNA target.
3. Polymerisation of a new DNA strand is initiated from the primers, and once the polymerase reaches the probe, its 5'-3'-exonuclease degrades the probe, physically separating the fluorescent reporter from the quencher, resulting in an increase in fluorescence.
4. Fluorescence is detected and measured in the real-time PCR thermocycler, and its geometric increase corresponding to exponential increase of the product is used to determine the threshold cycle (C_T) in each reaction.

2.4.2.2.5 Quantification

Concentrations of DNA present during the exponential phase of the reaction are determined by plotting fluorescence against cycle number on a logarithmic scale (so an exponentially increasing quantity will give a straight line). A threshold for detection of fluorescence above background is determined. The cycle at which the fluorescence from a sample crosses the threshold is called the cycle threshold, C_t . The quantity of DNA theoretically doubles every cycle during the exponential phase and relative amounts of DNA can be calculated, e.g. a sample whose C_t is 3 cycles earlier than another's has $2^3 = 8$ times more template. Since all sets of primers do not work equally well, one has to calculate the reaction efficiency first. Thus, by using this as the base

and the cycle difference C_t as the exponent, the precise difference in starting template can be calculated (in previous example, if efficiency was 98%, then the sample would have $2^{(3 \cdot 98\%)} = (2^{98\%})^3 = 7.67$ times more template.

Amounts of RNA or DNA are then determined by comparing the results to a standard curve produced by real-time PCR of serial dilutions (e.g. undiluted, 1:4, 1:16, 1:64) of a known amount of RNA or DNA. Real-time PCR can be used to quantify nucleic acids by two methods: relative quantification and absolute quantification. Relative quantification is based on internal reference genes to determine fold-differences in expression of the target gene. Absolute quantification gives the exact number of target DNA molecules by comparison with DNA standards (³⁶²Dhanasekaran *et al.*, 2010). There are numerous applications for real-time PCR in the laboratory. It is commonly used for both diagnostic and basic research. Diagnostic real-time PCR is applied to rapidly detect nucleic acids that are diagnostic of, for example, infectious diseases, cancer and genetic abnormalities. The introduction of real-time PCR assays to the clinical microbiology laboratory has significantly improved the diagnosis of infectious diseases (³⁶³Sails, 2009), and is deployed as a tool to detect newly emerging diseases, such as flu, in diagnostic tests (³⁶⁴FDA News, April 27, 2009).

Figure 1. Application of the amplification curve in real-time PCR

Amplification Curves

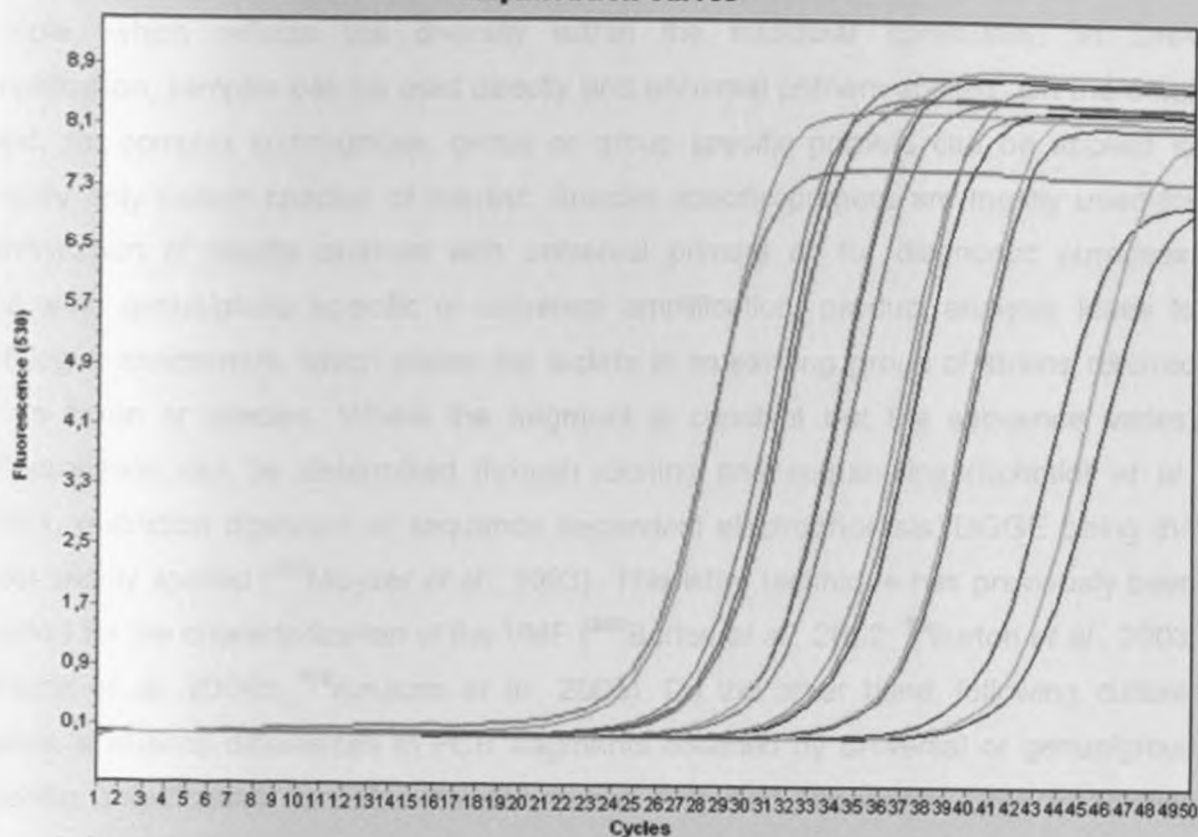


Figure 15- Illustration of the amplification curves in real time PCR

2.4.2.2.6 PCR-based Culture-independent Techniques

PCR-based methods can also be applied to identify the bacteria species composition of complex communities. The result is usually a profile of nucleic acids amplified from a sample, which reflects the diversity within the microbial community. In DNA amplification, samples can be used directly and universal primers applied. On the other hand, for complex communities, genus or group specific primers can be applied to amplify only certain species of interest. Species-specific primers are mostly used for confirmation of results obtained with universal primers or for diagnostic purposes. Following genus/group specific or universal amplification, product analysis leads to catalogue assignment, which places the isolate in an existing group of strains referred to as taxon or species. Where the fragment is constant but the sequence varies, differentiation can be determined through cloning and sequencing (Schmidt *et al.*, 1991), restriction digestion or sequence dependent electrophoresis, DGGE being the most widely applied (³⁶⁵Muyzer *et al.*, 1993). This latter technique has previously been applied for the characterization of the VMF (³⁶⁶Burton *et al.*, 2002; ⁹⁵Burton *et al.*, 2003; ²⁸Ferris *et al.*, 2004b; ¹⁰²Anukam *et al.*, 2005). On the other hand, following culture, isolate sequence differences in PCR fragments obtained by universal or genus/group specific amplification can also be determined Amplified Ribosomal DNA Restriction Analysis (ARDRA). To simplify the resulting restriction patterns, only one of the PCR primers is labeled, to help visualise the terminal restriction fragment after capillary electrophoresis. Consequently, this technique has come to be known as Terminal Restriction Fragment Length Polymorphism analysis (T-RFLP) (³⁶⁷Liu *et al.*, 1997). This technique has also been applied to determine the diversity of the VMF (³⁶⁸Coolen *et al.*, 2005).

CHAPTER THREE

3.1 MATERIALS AND METHODS

3.2 RECRUITMENT, SCREENING AND FOLLOW-UP

3.2.1 Study Site

Tigoni District Hospital is situated in Kiambu West district in the Limuru highlands, being one of the 47 districts of Central Province. The district covers an area of 1,095km² and has a population projected to be 517,826. The institution was put up by British settlers in 1939 as a cottage hospital meant for the soldiers who had taken part in the Second World War. It remained a cottage hospital until 1993, when it was upgraded to a sub-district hospital and later in 2007 to a District Hospital. It serves as a referral centre to all the other 17 health centres and dispensaries operated by the Kenya Ministry of Medical Services and hence serves the whole district population. Kiambu West district borders Nairobi county to the North and the hospital is 40 km north-west of Nairobi .

The Tigoni District Hospital is a 65-bed facility whose catchment population are mainly employees of local horticultural farms, peasant farmers and traders. The hospital runs both in-patient and out-patient services and has a monthly attendance of 6,000 persons, including children. The outpatient services include routine laboratories, maternal and child health clinic and HIV/AIDS prevention and treatment programs, among others.

The Ministry of Health in Kenya holds a health policy that emphasizes control and prevention of HIV infection as well as control of other diseases, and seeks to promote research aimed at improving the health of its population. It is against such a background that every district has several primary health care clinics where residents can access medical care. In addition, as a way to curb the spread of HIV infection in Kenya, voluntary counselling and testing centres (VCT) have been set up in most health facilities in the country. Tigoni District Hospital has an active VCT centre serving about 20 clients per day. This makes it an ideal site for a study of this nature. Moreover, the hospital has a longstanding history

of collaboration with the University of Nairobi both in the areas of health care provision and in research.

3.2.2 Study Design

The Mucosal Immunity Study (MIS) was a cross-sectional study nested on an ongoing Tigoni Dysplasia Study (TDS) based at Tigoni Hospital, Kiambu West district, Kenya in 2007. The following protocol was followed to create a database for selection of patients.

From an existing database of about 6,000 women, a second database of 1,000 women was created starting from the most recent participants of the TDS study. The inclusion criteria for the MIS study summarized below were used to capture suitable participants from the population pool (Appendix 6):

- a) Either HIV positive or HIV negative by ELISA.
- b) Normal pap-smear test within the last one year
- c) CD₄ counts above 250 cells/ μ L for HIV positive cases.
- d) Age between 18 and 45 years.
- e) Not using any other contraceptive method except the condom.
- f) Not on anti-retroviral (ART) therapy.
- g) Not pregnant
- h) Had the last two regular menstrual cycles.

3.2.3 Sample size

Sample size was calculated using the formula shown below (³⁶⁹Delucchi, 2004).

$$Ss = \frac{Z^2 \times (p) \times (1-p)}{c^2}$$

Where:

Ss= sample size

Z = Z value (1.96 for 95% confidence level).

p = prevalence of HIV in Tigoni area in the period of the study was approximately

17% (³⁷⁰MOH, 2001), expressed as a decimal.

c = Margin of error (which is 0.05)

Eligible women were recruited consecutively until the required sample size of 215 was attained. Thereafter 100 women fulfilled the inclusion criteria, 50 of them HIV-ve and 50 HIV+ve. Additional HIV positive women were recruited from the voluntary counselling and testing centre (VCT) on site. All patients from the VCT who tested HIV+ve by the Rapid test method had the HIV test repeated using the conventional ELISA methods.

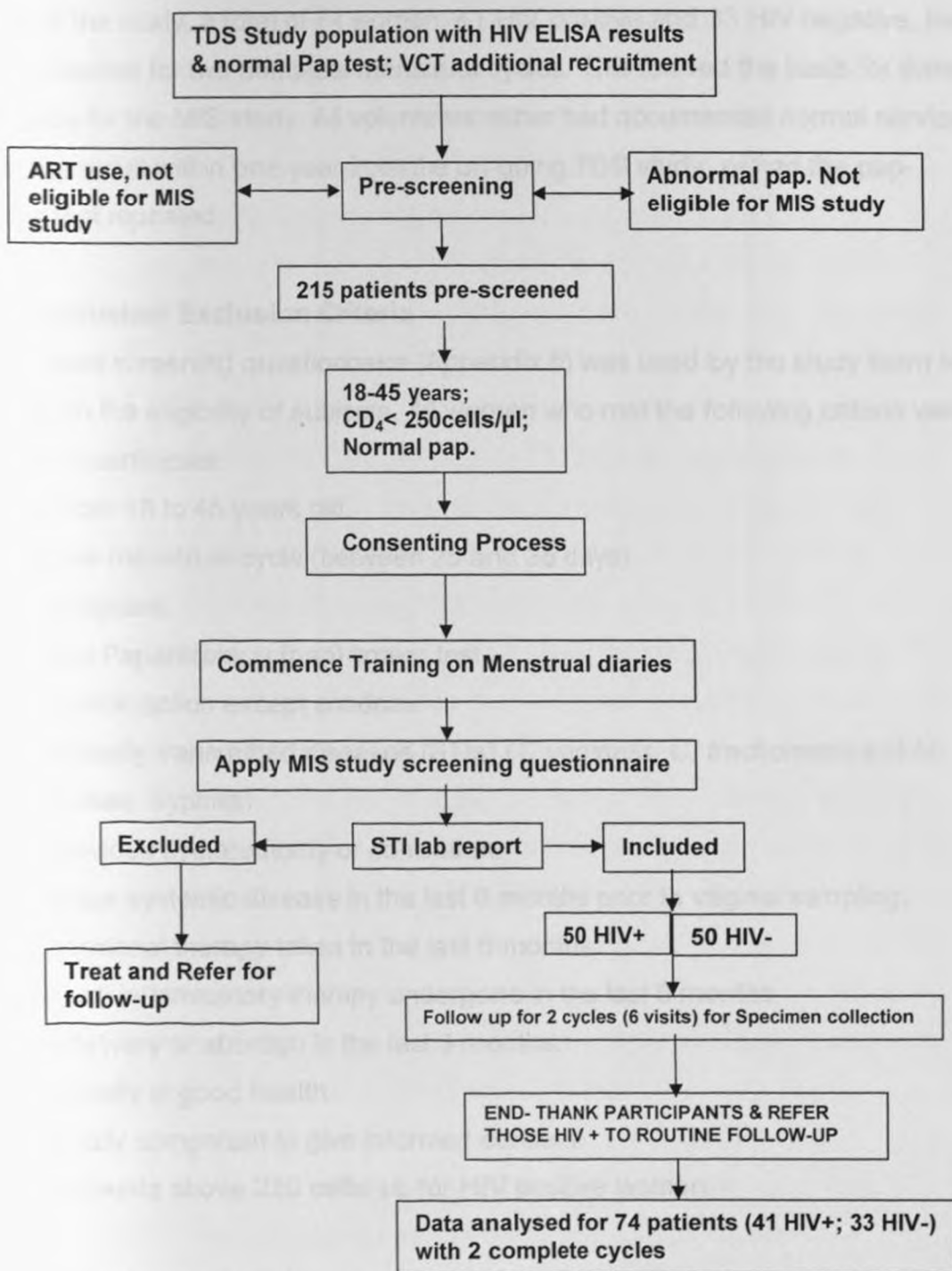
Other factors considered in the creation of the database were accessibility of patients as assessed through tracer/ contact information and their willingness to participate in the study. They also had to be available to keep appointments within the two-month duration of the study. Patients who met the selection criteria were initiated into training on use of menstrual diaries. Those who did not have a documented normal pap smear result in the previous one year had the test carried out according to the approved protocol (Appendix 15).

3.2.4 Training on Menstrual Diaries

Eligible women were invited to participate in the MIS study. Those who expressed willingness to participate were commenced on training in use of menstrual diaries (Appendix 16a and 16b). This exercise which took place two weeks before laboratory screening, helped to identify women who did not have regular menses. This group would not have been identified through the screening questionnaire alone.

All patients who qualified as per the above menu were trained on use of menstrual diaries in order to mark from home the days of menses and also be able to follow the appointment schedule, which was very stringent.

Figure 16: Summary of MIS Patient Recruitment Frame



3.2.5 Recruitment of Patients

Of the 215 women screened, a total of 100 (50 HIV positive and 50 HIV negative) premenopausal women were consecutively recruited for the MIS study. At the close of the study, a total of 74 women, 41 HIV positive and 33 HIV negative, had data collected for two complete menstrual cycles. This formed the basis for data analyses for the MIS study. All volunteers either had documented normal cervical cytology result within one year from the on-going TDS study, or had the pap-smear test repeated.

3.2.6 Inclusion/ Exclusion Criteria

A standard screening questionnaire (Appendix 6) was used by the study team to decide on the eligibility of subjects. All women who met the following criteria were invited to participate:

1. Age from 18 to 45 years old.
2. Regular menstrual cycle (between 25 and 35 days).
3. Not pregnant.
4. Normal Papanicolaou (pap) smear test.
5. No contraception except condom.
6. No sexually transmitted diseases (STIs) (*T. vaginalis*, *C. trachomatis* and *N. gonorrhoeae*, Syphilis).
7. No previous hysterectomy or conisation.
8. No major systemic disease in the last 6 months prior to vaginal sampling.
9. No hormonal therapy taken in the last 6 months.
10. No anti-inflammatory therapy undergone in the last 6 months.
11. No delivery or abortion in the last 3 months.
12. Generally in good health.
13. Mentally competent to give informed consent.
14. CD₄ counts above 250 cells/ μ L for HIV positive women.

Following a face-to-face interview and consent to participate in the study, subjects were requested to provide blood specimens for the HIV testing and CD₄

count. The tests results were subsequently used to categorise the subjects into two groups, namely HIV positive and HIV negative.

All patient data were recorded and an appointment date set depending on the monthly cycle of each study participant. Specimens were collected at three time points during the menstrual cycle – follicular, ovulation and luteal phases. Each phase was spread out within four days to allow flexibility for return visits. As a reminder to the next clinic visit, an appointment card was issued to each participant. They were also expected to use the card to mark all the days of menstrual bleeding as a guide to establishing regularity of the cycle (Appendix 16a and 16b).

3.2.7 Informed Consent of Participants and Ethical Considerations

A comprehensive questionnaire prepared for the purpose of the study was intended to provide information on specific areas. Prior to giving informed consent, participants were given an elaborate explanation of MIS study which included details of the study objectives. Thereafter, they voluntarily signed an informed consent form (Appendix 3 and 4). The benefits and risks associated with participation as well as the assurance of confidentiality in the handling and storage of all information and specimens provided by study clients was clearly explained (Appendix 1 and 2). The consent form and MIS study explanation were available in both English and the local Kiswahili language, and participants took their preferred choice for best communication. Only those women who met the recruitment criteria and gave consent to participate were enrolled for the study (Appendix 6) and had screening specimens taken. They also provided contact information (Appendix 5) for later follow up (Appendix 9 and 10). Enroled participants were followed-up rigorously to minimise loss and ensure sustainability of the study. Home visits and telephone calls were the main avenues to maintain constant contact throughout the study duration.

The principles of research on human subjects were applied at all times during the study. However, special consideration was exercised relating to HIV research and standard of care for patients who are infected with HIV virus. The study explanation form included extra details aimed at fostering a clear understanding with the participants. The participants were free to return on unscheduled visits if they had a health need (Appendix 11) and clinical notes taken on all visits were kept in the confidential patient files (Appendix 12). The files also had an attachment for recording of serious adverse events (Appendix 13) and any occurrence of protocol violation (Appendix 14).

3.2.7 Clinical Examination and Collection of Specimens

The standard requirements by the Ministry of Health for pre-test and post-test counselling and referral for appropriate care were observed throughout the study duration. The study protocol was approved by the Kenyatta National Hospital Research and Ethics Committee, a body charged with the responsibility of reviewing all research activities undertaken by staff and students as well as other institutions affiliated to the University of Nairobi. The study registration number is P122/8/2005. Permission was granted by the Tigoni hospital Management Board.

3.2.8 STI and HIV Testing and Treatment

Any STI diagnosed was treated according to Ministry of Health Kenya national guidelines. All HIV+ve women received treatment free of charge, sponsored by the partnership of Kenya Ministry of Health, University of Nairobi Institute of Tropical and Infectious Diseases and Pathfinder International. Further all HIV+ve persons with a CD₄ count above 200 cells/ μ L were, at the time of the study, exempted from ART (⁴²WHO, 1997).

3.2.9 Sampling and Laboratory Procedures

3.2.8 Sampling Regimen

On subsequent follow-up visits an interval history was obtained for any symptoms, the use of vaginal products and antibiotics, as well as sexual activity. All the women were asked to abstain from sexual intercourse and use of vaginal products such as spermicides, douche or other topical agents for 3 days prior to

insertion of the device and removing it carefully to avoid contamination with

sample collection because these conditions may affect the composition of microflora in the cervicovaginal canal (⁵Wilson, 2005).

Specimens were collected three times per cycle for two consecutive menstrual cycles as follows:

1. Follicular phase (Day 5-8)
2. Ovulation phase (Day 12-15)
3. Luteal phase (Day 19-22)

3.2.9 Clinical Examination and Collection of Specimens

3.2.9.1 Patient Examination

Each patient underwent a general physical examination at which the following parameters were measured: pulse rate, arterial blood pressure, body weight and height.

Thereafter, the subject was asked to lie in the standard position for gynaecological examination with feet in stirrups, and a sterile non-lubricated vaginal speculum inserted. The clinician/ nurse performed a thorough pelvic examination to confirm normal anatomy and collect specimens. Inspection of the vulva, vagina and cervix was performed for visible signs of inflammation. Women with visible signs of genital infection were excluded from the study and referred for further diagnosis and treatment.

3.2.9.2 Screening Specimens (Appendix 21)

A specially trained project nurse collected a venous blood sample using aseptic technique. Screening blood specimens were used for the following tests: (1) Plasma for CD₄ cell count; and (2) serum for HIV ELISA and Syphilis sero-reactivity test.

Endocervical and high vaginal swabbing

The endocervical sampling was carried out by gently rotating a cotton swab in the opening of the cervix and removing it carefully to avoid contamination with

the vulva and introitus microflora. The swabs were transported to the laboratory in dry sterile tubes for STI diagnosis. One endocervical swab was used for both *N. gonorrhoeae* and *C. trachomatis* PCR. Two high vaginal swabs were collected. One was used for diagnosis of *T. vaginalis* by wet prep and culture and the other for preparation of gram stain.

Patients whose laboratory results met the inclusion criteria were then enrolled in the study and followed up according to the study protocol (Appendix 7 and 8).

3.2.9.3 Follow-up specimens

The pH of the vagina was determined by placing Color-pHast indicator strips (EM Science, Gibbstown, NJ) on the wall of the posterior fornix of the vagina and reading it against the provided standard.

With a non-lubricated speculum in place three cotton swabs were inserted into the vaginal vault consecutively. Each swab was rotated against the vaginal wall at the mid-portion of the vault and removed carefully to avoid contamination with the vulva and introitus microflora. One swab was returned each to a sterile transport tube (Copan, Brescia, Italy) for DNA extraction. The second swab was placed in Amies transport medium (Nuova, Aptaca, Canelli, Italy) for anaerobic culture and processed in the microbiology laboratory within 4 hours of collection. The last swab was used for making a Gram stain preparation on a frost-ended slide. The slides were labelled with patient's study number and initials, date and visit number prior to specimen collection.

3.3 SOPS FOR SCREENING LABORATORY TESTS

Following the collection of specimens at the field site to carry out laboratory-based screening of study participants, the following SOPs were applied to process them. Based on the results obtained, a patient was either excluded and referred for treatment, or enrolled in the study for follow up and specimen collection. The SOPs applied were the following:

3.3.1 GENERAL INSTRUCTIONS

Universal Precautions

Since a medical history and physical examination cannot identify all patients with infectious agents, standard precautions were consistently used for all patients.

The blood, body fluids and other specimens of every patient were considered to be potentially infectious. Thus, protective laboratory wear was used at all times whilst handling specimens.

Blood collected in EDTA tubes was used for the differential and CD₄ cell counts.

The anticoagulant EDTA was preferred to the contemporary heparin, since the former is known to be more stable compared to the latter. Heparin has been shown to induce apoptosis (³⁷¹Manaster *et al.*, 1996), and the resulting lysis could alter the test result.

Using a 'vacutainer' tube 5 ml each of plasma and serum were drawn consecutively. The clot in serum was sedimented and removed by centrifugation and the serum transferred into labelled plastic vials for the HIV ELISA test. The plasma was used for CD₄ counts.

3.3.2 SOP HIV-1 ELISA TEST

3.3.2.1 Background and principle

The diagnosis of HIV relies on the detection of the particular antibodies in the circulation or in tissues. Serum was tested for HIV-1 antibodies using a synthetic peptide enzyme immunoassay (ELISA, Detect HIV-1, Bioch Immunosystems, Inc., Montreal, Canada).

The ELISA was run in the microbiology laboratory of the Department of Medical Microbiology, Kenyatta National Hospital, Nairobi. Women who tested positive on the initial screening test had a confirmatory test carried out using Vironostika HIV-1 Plus Microelisa System (BioMerieux Inc, Durban, NC).

Two ml of the blood was placed in separate serum tubes for the HIV test. The blood sample for the test was stored at fridge temperature (2-8 °C) and the ELISA carried out within two days of collection.

3.3.2.2 Procedure for HIV ELISA (Detect HIV-1 kit)

1. Mix 250 µl sample diluent with 50 µl test sample in a microplate.
2. Take 100 µl of the mixture and add to strips precoated with HIV-1 p24 antigen.
3. Leave 3 wells of negative control and 3 wells of positive control.
4. Mix the contents and incubate at room temperature for 30 min.
5. Dilute conjugate accordingly and add 150 µl to each well.
6. Incubate in the dark for 30 min.
7. Add 100 µl of 1M sulphuric acid to stop the reaction.
8. Read the optical density (OD) at 450 nm, using 650 nm as the reference point.

Add the 3 readings of the negative control to get the average. Thereafter, add the constant provided in the kit manual to get the cut-off point. Values above the cut-off point indicate positive for HIV antibodies and those below indicate a negative test result.

3.3.3 SOP PAP SMEAR PROCEDURES

3.3.3.1 Background and principle

Pap smear is a screening test used to find abnormal cell changes that arise from cancer of the cervix or its precursor lesions (before cervical cancer develops).

The Pap smear involves collection of a sample of cells from the uterine cervix using a spatula or brush, smeared onto a slide and examined under a microscope for abnormal cells (pre-cancer or cancer). Devices used for Pap smear sampling are wooden spatula (Ayre's spatula), endocervical brush or plastic brush/broom. When a Pap smear shows abnormal result it is referred to as positive. Women with a positive Pap smear need more tests to confirm the diagnosis and to determine the diagnosis and the treatment required.

Since the mid-1990s, techniques based on placing the sample into a vial containing a liquid preservative for cells have increasingly been applied. The media are primarily ethanol based and Thin-Prep (Cytoc Corp) was used. Once placed into the vial, the sample is processed at the laboratory into a cell-thin layer, stained, and examined by light microscopy. The liquid sample has the advantage of being suitable for low and high risk HPV testing and reduced unsatisfactory specimens from 4.1% to 2.6% (³⁷²Ronco *et al.*, 2007). Proper sample acquisition is crucial to the accuracy of the test as a cell that is not in the sample cannot be evaluated.

Studies of the accuracy of liquid based monolayer cytology report:

- Sensitivity 61% (³⁷³Kulasingam *et al.*, 2002) to 66% (³⁷⁴Coste *et al.*, 2003)
- Specificity 82% (³⁷³Kulasingam *et al.*, 2002) to 91% (³⁷⁴Coste *et al.*, 2003)

Some (³⁷²Ronco *et al.*, 2007), but not all studies (³⁷⁴Coste *et al.*, 2003; ³⁷³Kulasingam *et al.*, 2002) report increased sensitivity from the liquid based smears.

The Bethesda System is a system for reporting cervical or vaginal cytologic diagnoses and for reporting Pap smear results. It was introduced in 1988, and revised in 1991 and 2001 (³⁷⁵Strander, 2009). The name comes from the location (Bethesda, Maryland) of the conference that established the system.

Abnormal results include:

- Atypical squamous cells of undetermined significance (ASCUS)
- Atypical squamous cells - cannot exclude High grade squamous intraepithelial lesion (ASC-H)
- Low grade squamous intraepithelial lesion (LGSIL)
- High grade squamous intraepithelial lesion (HGSIL)
- Squamous cell carcinoma

- Atypical Glandular Cells not otherwise specified (AGC-NOS)
- Atypical Glandular Cells, suspicious for Adenocarcinoma *in situ* (AIS) or cancer (AGC-neoplastic)
- Adenocarcinoma *in situ* (AIS)

3.3.3.2 Procedure

Sample collection

The procedure used for Pap smear collection was as follows:

1. Label the Papsin collection fluid bottle with the patient's details.
2. With a speculum in place, examine the cervix for any abnormalities such as erosion, inflammation, discharge or masses. Record findings on patient file.
3. Insert the longer, central part of the broom into the cervical os and brush the shorter, outer part against the ectocervix.
4. While maintaining gentle pressure in the direction of the cervix, rotate the brush 5 times in either direction.
5. Immediately remove the white bristle end of the Papette brush from its handle and drop into the vial of Papsin collection fluid.
6. Cap the vial tightly and shake to dislodge the epithelial cells into the medium.
7. Prepare all samples for transport to the histology laboratory, each accompanied by an order form.

3.3.3.3 Lab processing and reporting

- a) Vortex for 2 minutes to dislodge the cells from the brush and homogenize the fluid.
- b) **Cytospin** to make a monolayer of the cell suspension on the microscope slide.
- i) On the assembled cytospin cuvette put 3.0 mls of the homogenized cell suspension.

ii) Run the cytospin machine at 2500 rpm for 5 minutes.

iii) Fix in 95% Ethanol for 10 minutes.

(If the cell suspension is too heavy it can be diluted in 95% ethanol)

c) Staining Procedure

i) Immerse the smear in 95% ethanol for 15 minutes to remove the carbol wax present in the transport kit preservative. Rinse in water.

ii) Stain with heamatoxylin for 3 minutes. Rinse in water.

iii) Differentiate stain in 1% acid alcohol (1% HCl in 70% ethanol) for 4 seconds (or four dips). Rinse in water.

iv) Immerse slides in Scotts water for 4 seconds. This alkaline solution (magnesium sulphate and sodium hydrogen carbonate) neutralizes the acid from the previous step. Rinse in water.

v) Rinse in 70% alcohol for 10 dips.

vi) Rinse in 95% alcohol for 10 dips.

vii) Stain in Orange-G for 2 minutes.

viii) Rinse in two changes of 95% alcohol for 10 dips each.

ix) Stain in eosin azure for 3 minutes.

x) Rinse in three changes of 95% alcohol for 10 dips each.

xi) Rinse in three changes of 100% alcohol for 10 dips each.

xii) Rinse in three changes of xylene for 10 dips each.

xiii) Mount with DPX (distyrene plasticizer xylene) for examination.

3.3.3.4 Microscopy and reporting

Screening is done at X100 magnification and confirmation for suspicious cells at X 400.

Reporting of the results was done by the pathologist using a standard form specified for MIS study (appendix 15).

3.3.4 SOP SYPHILIS CARD TEST

3.3.4.1 Background and principle

The rapid plasma reagin (RPR)/Carbon Antigen test is a macroscopic non-Treponemal flocculation test for the detection and quantification of antilipoidal antibodies.

Syphilis is a chronic contagious and often congenital venereal disease caused by *Treponema pallidum*. This organism belongs to a gram-negative group of bacteria called the spirochetes, which are defined by their unique cellular structure. Infection results from contact with moist surfaces, originating in lesions of the epithelial tissue of the skin and mucous membranes. If untreated the disease may result in irreversible changes in the cardiovascular and nervous systems.

The TPHA test was used as a confirmatory test for the syphilis RPR test.

Syphilis RPR is a non-treponemal flocculation test used to detect and quantify reagin antibodies associated with syphilis.

3.3.4.2 Procedure

The RPR – antigen used contains microparticulate charcoal to enhance the visual difference between a positive and a negative result. For sera containing anti-reagin antibodies flocculation of antigen-charcoal particles is observed. In case of non-reactive specimen the specimen-antigen-suspension remains homogenous.

Confirmatory *Treponema pallidum* haemagglutination assay was not necessary as no seroreactive cases were detected.

3.3.5 SOP CHLAMYDIA TRACHOMATIS PCR TEST

3.3.5.1 Background and principle

Chlamydia trachomatis is an obligate intracellular parasite that preferentially infects endocervical columnar cells present in the ectocervix (³⁷⁶Jacobson *et al.*, 2000). Three species exist: *C. trachomatis*, *C. psittaci* and *C. pneumoniae*. There are 15 serovars of *C. trachomatis* namely, A, B and C which are found in areas of

endemic trachoma. In the sexually transmitted disease, *C. trachomatis* causes urethritis, cervicitis, pelvic inflammatory disease, endometritis and ophthalmia neonatorum. Serovars L₁, L₂ and L₃ cause lymphogranuloma venereum, a lymphadenopathy of the inguinal region (³⁷⁷WHO, 2005).

3.3.5.2 Procedure

An endocervical swab is usually taken for aetiological diagnosis of cervicitis, diagnosis of *N. gonorrhoea* and *C. trachomatis* infection in women. Diagnosis is made by microscopic examination, PCR and/or culture. It must be noted however, many women have non-specific symptoms or no symptoms at all. Thus, diagnosis can be made more accurately through additional confirmatory tests such as culture and/ or PCR (³⁷⁸WHO, 1995).

3.3.5.2.1 Specimen collection

1. In case of any discharge around the endocervix wipe it off with gauze or with a swab and discard the used swab.
2. Insert a clean swab into endocervix until most of the tip is no longer visible and rotate firmly 3-4 times against the walls of the endocervix.
3. Place the swab in labeled transport tube and break off the shaft in order to cap the tube tightly.
4. Transport the swabs to the laboratory with the appropriate request forms completely filled with patient details (³⁷⁹NCK, 1998; Appendix 6).

3.3.5.3 Laboratory processing

Chlamydia trachomatis and *N. gonorrhoeae* samples were tested using a standardised commercial PCR test (Cobas Amplicor, Roche Diagnostics, USA).

3.3.5.3.1 Extraction procedure:

1. Reconstitute the dry swab in 1000 µl of 2.0 M sucrose.
2. Mix 100 µl of the bacterial suspension with 100 µl lysis buffer. Keep the remaining 800 µl for repeat runs if necessary.
3. Vortex the mixture thoroughly and incubate for 10 minutes at room temperature.
4. Add 200 µl MgCl₂ to a final concentration of 3.0 M, giving a total volume of 400 µl. Vortex to mix.

3.3.5.3.2 Amplification

5. Prepare reaction map for the specimens.
6. To 1.8 ml master-mix, add 100 µl Manganese ions and mix gently to avoid foaming.
7. Pick 50 µl of the above mixture from a trough using 8-way multichannel pipette into reaction tubes.
8. Add 50 µl of specimen and mix. Replace caps to avoid contamination and then proceed to next row.
9. Put the reaction tubes in the pre-set thermocycler (Takes approx. 2 hours).

3.3.5.3.3 Detection

10. During the final hold program at 72 °C, add 100 µl denaturation solution (NaOH, EDTA, Thymol blue) into the reaction tubes. This denatures the amplified DNA product into single strands.
11. Specific detection plates for Chlamydia and *N. gonorrhoeae* are used.
12. From a mixing trough put 100 µl of the denaturation solution into the wells of the detection plates.
13. Add 25 µl of the PCR product and mix by gently tapping the plate.
14. Place loose cover on the plate and incubate at 37 °C for 60 minutes.
15. Discard the well contents and blot dry onto absorbent towels.
16. Wash 5 times with the reconstituted kit wash buffer.
17. Blot the plates dry.

18. Add 100 µl conjugate (Avidin-horseradish peroxidase) and incubate at 37 °C for 15 minutes.
19. During this incubation prepare the working substrate and store in the dark.
20. After the incubation pour out the plate contents and blot dry.
21. Wash 5 times with the reconstituted kit wash buffer.
22. Add 100 µl of the working substrate into each well and store in the dark for 10 minutes to allow for colour development.
23. Add 100 µl stop solution (Sulfuric acid).
24. Measure absorbance at 450nm within 1 hour of adding stop solution.
25. Record each specimen absorbance and the accompanying control.
26. All positive are repeated for confirmation.

3.3.6 SOP NEISSERIA GONORRHEA PCR TEST

3.3.6.1 Background and principle

Neisseria gonorrhoeae, the causative agent of gonorrhoea, is a gram-negative, intracellular diplococcus. It is kidney-shaped with adjacent concave sides. *N. gonorrhoeae* is strictly a parasite of man. It causes urethritis in males and pelvic inflammatory disease in women. It is also one of the causes of ophthalmia neonatorum in children.

Diagnosis involves microscopy (Gram stain, observed with light microscope with 100X objective), culture, biochemical and serological tests.

3.3.6.2 Procedure

Endocervical swab was transported to the lab in a sterile dry tube.

Neisseria gonorrhoeae was detected by the same PCR test used for Chlamydia (SOP 3.3.5).

3.3.7 SOP TRICHOMONAS VAGINALIS CULTURE

3.3.7.1 Background and principle

Trichomonas vaginalis is a pathogenic flagellate that infects the urogenital tracts of males and females. It is primarily an STI. Infection in females can result in

vaginitis, cervicitis, and urethritis. In males, the most common symptomatic presentation is urethritis. Diagnosis is by microscopy and culture.

3.3.7.2 Procedure

3.3.7.2.1 Specimen collection

1. With the speculum in place, insert the cotton swab and rotate it three times against the vaginal wall at the mid-portion of the vault and remove carefully to avoid contamination with the vulva and introitus microflora. Without touching the swab against anything, insert it in the In-Pouch culture tube.

- To avoid fluid leakage, move the medium at the top of the In-Pouch downwards to the middle of the upper chamber.
- Tear off plastic above white closure.
- To insert swab, open In-Pouch by pulling the closure tape's middle tabs apart.
- Obtain specimen on a cotton swab.
- Insert swab into upper chamber medium and elute the specimen by rubbing swab between the In-Pouch walls.
- Remove the swab and discard
- Squeeze the closure strip with the thumb and forefinger of one hand.
- Hold the bottom of the pouch with the other hand and move the medium from the top chamber to the lower chamber by pulling it upwards across the edge of the counter/table in a "shoe shine" motion
- Now roll the empty upper chamber down to the top of the label. Fold the tabs over to prevent the In-Pouch from reopening.
- Label and store the In-Pouch for transport to the laboratory.

2. Similarly collect the second high vaginal swab for microscopic examination.

3.3.7.2.2 Microscopy

Plain slides, cover slips, normal saline, HVS, Microscope, Record sheet, pen.

To make a saline wet preparation the specimen is suspended by rolling the high vaginal swab in a drop of saline on a slide. Place the cover slip.

Examine at 100X magnification and look for typical jerky movement of motile trichomonads.

Alternatively 1 to 2 drops of cervicovaginal fluid placed on a slide can be examined microscopically for detection of motile trichomonads.

3.3.7.2.3 Culture

Without touching the swab against anything, insert the swab into the In-Pouch TV (Biomed diagnostics Inc, San Jose, CA) culture medium compartment and transport to the laboratory for anaerobic incubation at 37 °C for 5-7 days for the detection of motile trichomonads. Bio Med's In-Pouch TV (*T. vaginalis*) Culture System comprises a clear, plastic envelope containing culture medium. The medium is selective for the transport and growth of *T. vaginalis*, while inhibiting the growth of contaminating microorganisms that might interfere with a reliable result. The medium is inoculated with the vaginal fluid sample. The sample is incubated in the envelope, and the envelope is placed directly on the Microscope for reading. Transport and off-site testing can be performed easily due to the flexible packaging and design of the In-Pouch.

Requirements

Materials:

- In-Pouch device
- Swabs
- Gloves

Equipment:

- Light microscope
- Incubator 37 °C
- Viewing clamp

3.3.7.2.4 Specimen handling

Once inoculated and sealed, the In-Pouch TV medium can be kept up to 48 hours at room temperature if necessary. After 48 hours, incubation at 37°C is required.

Procedures:

- Specimens are received from the field at the end of each day (about 5 pm).
- Incubate at 37 °C on receipt of the specimen.
- Specimens are microscopically examined on the day following delivery to the laboratory (this is the 24 hour examination). Microscopic examination is also conducted at 48h, 72h and 96h using the procedure described below.

Procedures for microscopic examination in the laboratory:

- Ensure that all the media is in the lower pouch. If some of the media has moved into the upper pouch, do the following:
 - Squeeze the closure strip with the thumb and forefinger of one hand.
 - Hold the bottom of the pouch with the other hand and move the medium from the top chamber to the lower chamber by pulling it upwards across the edge of the counter/table in a “shoe shine” motion
 - Now roll the empty upper chamber down to the top of the label, fold the tabs over to prevent the In-Pouch from reopening.
- Place the viewing clip horizontally over the upper chamber.
- Close the clip.
- Place the pouch on the stage and scan under 10X and 20X.
- Confirm under 40X.

3.3.7.2.5 Tips for microscopic examination:

- Even though the In Pouches look like clear broth to the naked eye, under the microscope you will observe crystal –like debris floating about. This is not contamination but rather nutrients for the *T. vaginalis*.
- Trichomonads gravitate to the edges of the In-Pouch just as they gravitate to the edges of a cover slip on a standard wet mount.
- If none are seen along the edges, briefly scan the fluid.
- Focus on the fluid and not on the textured plastic of the pouch.
- The anti-fungal and anti-microbial ingredients in the In-Pouch inhibit yeast and bacteria from multiplying, they will be held “in stasis” for several hours. Yeast and clue cells may be observed.

As the trichomonads multiply, white sediment will be observed along the sides and the bottom of the chamber

- The medium can also change to a darker colour.

Notes:

- The medium should **NEVER** be centrifuged as to concentrate the trichomonads.
- The In-Pouch kit is for cultivation of *T. vaginalis* only. Others species of trichomonads will not survive and replicate at the pH and medium composition of the In-Pouch.
- Vaginal swabs may be collected during menses. Studies have shown no change in sensitivity and specificity of the test if the sample is collected during menses.

3.3.7.2.6 Interpretation of results:

- If trichomonads are seen on any of the microscopic exams, the results are recorded as positive and the specimen is discarded.
- If no trichomonads are seen on initial exams, continue to incubate and re-examine according to schedule above.

- If **NO** trichomonads are visualized after **96 hours (4 days)** and incubation at 37 °C, record result as negative and discard specimen.

Limitations

- *T.vaginalis* is a very sticky organism and will adhere to plastic containers, plastic tubes, plastic pipettes, Dacron swabs, condoms, and mucous.
- Use only cotton swabs, wire microbiological loops, glass containers, glass tubes and glass pipettes.

References

- Package insert
- Standard operating procedures on microscopic examination of *T. vaginalis* from University of Manitoba (UNIM) and CDC-KEMRI clinical diagnostics laboratory.

3.3.8. SOP VAGINAL CANDIDIASIS

3.3.8.1 Background and principle

Vaginal candidiasis was be diagnosed microscopically from a vaginal swab in the wet preparation with 10% potassium hydroxide to detect the presence of yeast cells. Budding yeast cells and filaments can be seen under the microscope. In the Gram stain large gram-positive yeast cells can be observed.

3.3.8.2 Procedure

Spread swab contents onto a clean labelled slide by rolling both sides at the centre of the slide. To make identification of yeast cells easier in wet mount slides, mix the vaginal swab in another drop of saline and add a drop of 10% potassium hydroxide to dissolve other cells.

Cover with a cover slip.

Examine at 400X magnification to look for yeast cells and trichomonads.

Specify if spermatozoa are seen or not.

3.3.9 SOP CD₄ COUNTS

3.3.9.1 Background and principle

Hematological investigation is an important part of disease diagnosis and provides useful information on erythroid, leukocytic and platelet/ thrombocytic alterations. Differential blood cell counts were done in the Hematology Laboratory at Kenyatta National Hospital, Nairobi, to identify and enumerate the blood cells.

The CD₄ count tells the doctor how strong the immune system is, how far HIV disease has advanced (the stage of the disease), and helps predict the risk of complications and debilitating infections. The CD₄ count is most useful when it is compared with the count obtained from an earlier test.

The CD₄ count is used in combination with the viral load test to determine the staging and outlook of the disease. It is also used to identify possible health problems for which one may be at risk and to determine which medications might be helpful. A CD₄ count and a viral load test are ordered when a person is first diagnosed with HIV as part of a baseline measurement.

3.3.9.1.1 Significance of the CD₄ test result

A standard reference range is not available for this test. Because reference values are dependent on many factors, including patient age, gender, sample population, and test method, numeric test results have different meanings in different labs. Each lab report should include the specific reference range for its test. Normal CD₄ counts in adults range from 500 to 1,500 cells per cubic milliliter of blood.

According to public health guidelines, preventive therapy should be started when an HIV-positive person who has no symptoms registers a CD₄ count under 200 (379 WHO, 1995). The Centers for Disease Control and Prevention considered

HIV-infected persons who had CD₄ counts below 200 to have AIDS, regardless of whether they are sick or well.

The objective of the test is to detect the immuno status of the patient by enumerating the absolute cell counts of CD₄, CD₈, and CD₃ T lymphocytes in unlysed whole blood.

It is the responsibility of all the laboratory technicians and other staff to follow this SOP.

The BD FACSCCount system is an automated instrument and reagent kit designed specifically for enumerating the absolute cell counts of CD₄, CD₈, and CD₃ T- lymphocytes in unlysed whole blood.

When whole blood is added to the reagents, fluorochrome-labeled antibodies in the reagents bind specifically to lymphocyte surface antigens. After a fixative solution is added to the reagent tubes, the sample is run on the instrument. Here, the cells come in contact with the laser light, which causes the fluorochrome-labeled cell to fluoresce. This fluorescent light provides the information necessary for the instrument to count the cells.

In addition to containing the antibody reagent, the reagent tubes also contain a known number of fluorochrome-integrated reference beads. These beads function as a fluorescence standard for enumerating the cells.

Analysis is automatic. The software identifies the T-lymphocyte populations and calculates the absolute counts. Results print immediately after samples are run and absolute cell counts for CD₄ (helper/inducer T lymphocytes, CD₈ (suppressor/ cytotoxic T lymphocytes), and CD₃ (total T lymphocytes), as well as CD₄/CD₈ (helper/suppressor T- lymphocyte ratio) are reported.

3.3.9.2 Procedure

3.3.9.2.1 Cell count

Plasma collected in EDTA tubes was centrifuged to remove the red blood cells. Plasma can be frozen below -80 °C nearly indefinitely for subsequent analysis. The flow cytometry using antibodies purchased from Becton & Dickson (San Jose, CA, USA) was done in the Department of Medical Microbiology, University of Nairobi. Stained samples will be analysed using a FacsCaliber instrument and CELLQuest Software (Becton & Dickson).

3.3.9.2.2 Collecting and preparing patient samples

Patient blood samples must meet the following criteria:

- . Blood must be collected in a K₃ EDTA (liquid) vacutainer tube.
- . Blood must be stored no longer than 48 hours at room temperature (20 °C to 25 °C).

N.B: Results obtained from samples that do not meet these criteria can be inaccurate.

Patient samples are prepared by adding blood, then fixative solution, to the CD₄ and CD₈ reagent tubes.

3.3.9.2.3 Specimen Preparation

- Label the tab of one reagent tube pair with the patient accession number or number that identifies the tube of blood.
- Vortex the pair upside down for 5 seconds.
- Vortex the pair upright for 5 seconds.
- Open the reagent tubes with the coring station.
- Mix the patient whole blood by inverting the tube five times.
- Pipette 50ul of the blood into each of the two reagent tubes. Change tips between tubes.
- Cap the tubes and vortex upright for 5 second.
- Incubate the tubes for 60 to 120 minutes at room temperature (20 °C to 25 °C).

- Place the reagent pair in the work station and close the cover to protect the reagents from light.
- Uncap the tubes and pipette 50 µl of fixative solution into each reagent tube. Change tips between tubes.
- Recap the reagent tubes with new caps and vortex upright for 5 seconds.
- Run the tubes on the FACS Count instrument within 48 hours of preparation.

3.3.9.2.4 Entering Patient and Reagent Information

- Enter an accession number for each sample before running the sample on the FACS Count instrument. The accession number is entered immediately before the sample is run.
- From the FACS Count screen or the CONTROL results screen, press [SAMPLE].
- This displays the REAGENTS screen, allowing one to enter or verify the reagent lot code and reference bead counts (see Entering control and Reagent Information).
- Enter or verify the reagent lot code and reference bead counts and press [CONFIRM].
- The SAMPLE screen is displayed.
- Enter the patient accession number (up to 15 characters).
- This is the laboratory number that identifies the tube of blood. If the accession number from the previous run exists, press [Clr Acc#] to remove it.
- The instrument prompts operator for the CD₄ tube (see Running Patient Samples).

3.3.9.2.5 Running Patient Samples

- Vortex the reagent pair upright for 5 seconds.
- Uncap the tube (green top) and set the cap aside. Place the reagent pair in the sample holder so the CD₄ tube is in the run position.

- Press [RUN]. A message appears informing you the test has started. This is referred to as a sample boost. When analysis of the CD₄ tube is complete, the sample holder lowers and the following message appears: [Move the CD₈ tube into position and press the RUN key. To abort run, press STOP.]
- Remove the reagent pair and recap the CD₄ tube. Uncap the CD₈ tube (clear top) and set the cap aside. Replace the pair so the CD₈ tube is now in the run position.
- Press [RUN]. The sample boost and acquisition / analysis status message that appeared for the CD₄ tube are displayed for the CD₈ tube. When analysis of the CD₈ tube is complete, the sample holder lowers.
- Remove the reagent pair and recap the CD₈ tube and discard the reagent pair in an appropriate biohazard container.
- The Patient results are displayed and printed.
- To run the next sample, press [SAMPLE], enter the patient accession number, and follow steps 1 through 6 above.

3.3.9.2.6 Preparing and Running Controls

- Collect blood from a normal blood donor in K₃ EDTA tube.
- Vortex for 5 seconds and open the controls (zero, normal, medium, high).
- Get two reagent pairs and label them zero, normal, medium, and high, vortex them upright and upside down for 5 seconds each.
- Open the reagent pairs and pipette 50 µl of normal blood into each tube and vortex for 5 seconds.
- Incubate the reagent pairs for 60 to 120 minutes in dark (workstation).
- Pipette 50ul of the zero control into one tube of the CD₄ and 50 µl of the normal into the CD₈ tube of one reagent pair.
- Pipette 50 µl of medium control and 50 µl of high control into the CD₄ and CD₈ tubes of another reagent pair respectively.
- Vortex the reagent pairs for 5 seconds.

- Run the controls on the FACS Count machine starting with the zero, normal, medium, and high tubes respectively.

3.3.9.2.7 Limitation of the test

- The CD₄ tube must be run before CD₈ tube.
- The samples must be collected in K₃ EDTA BD Vacutainer blood collection tube. A minimum of 200ul of whole blood is required for the test.
- Do not store the whole blood on a blood rocker or other mixing device.
- Do not refrigerate whole blood before preparing.
- Do not dilute whole blood or use any volume other 50ul.
- Store prepared samples at room temperature.
- The reagents used are light sensitive. Minimize exposing the reagent tubes to light.
- Do not rerun patient samples more than twice on the FACSCount after they have already been run.
- Use only BD FACSCount Reagents and controls with the BD FACSCount instrument.

3.4 MICROBIOLOGY METHODS

After laboratory screening and enrolment, study participants were followed up and high vaginal swab specimens taken for a total of six visits per woman, covering two consecutive menstrual cycles. The following SOPs were used to collect and process the swabs.

3.4.1 SOP COLLECTION OF SPECIMENS

3.4.1.1 Background and Principle

After screening for STIs and recruitment of study patients, follow-up specimens collected were mainly high vaginal swabs for preparation of gram stains, anaerobic culture and extraction of DNA for real time PCR.

3.4.1.2 Procedure

1. With a non-lubricated speculum in place 3 sterile cotton swabs were inserted consecutively into the vaginal vault above the cervix.

The swab was rotated three times against the vaginal wall at the mid-portion of the vault.

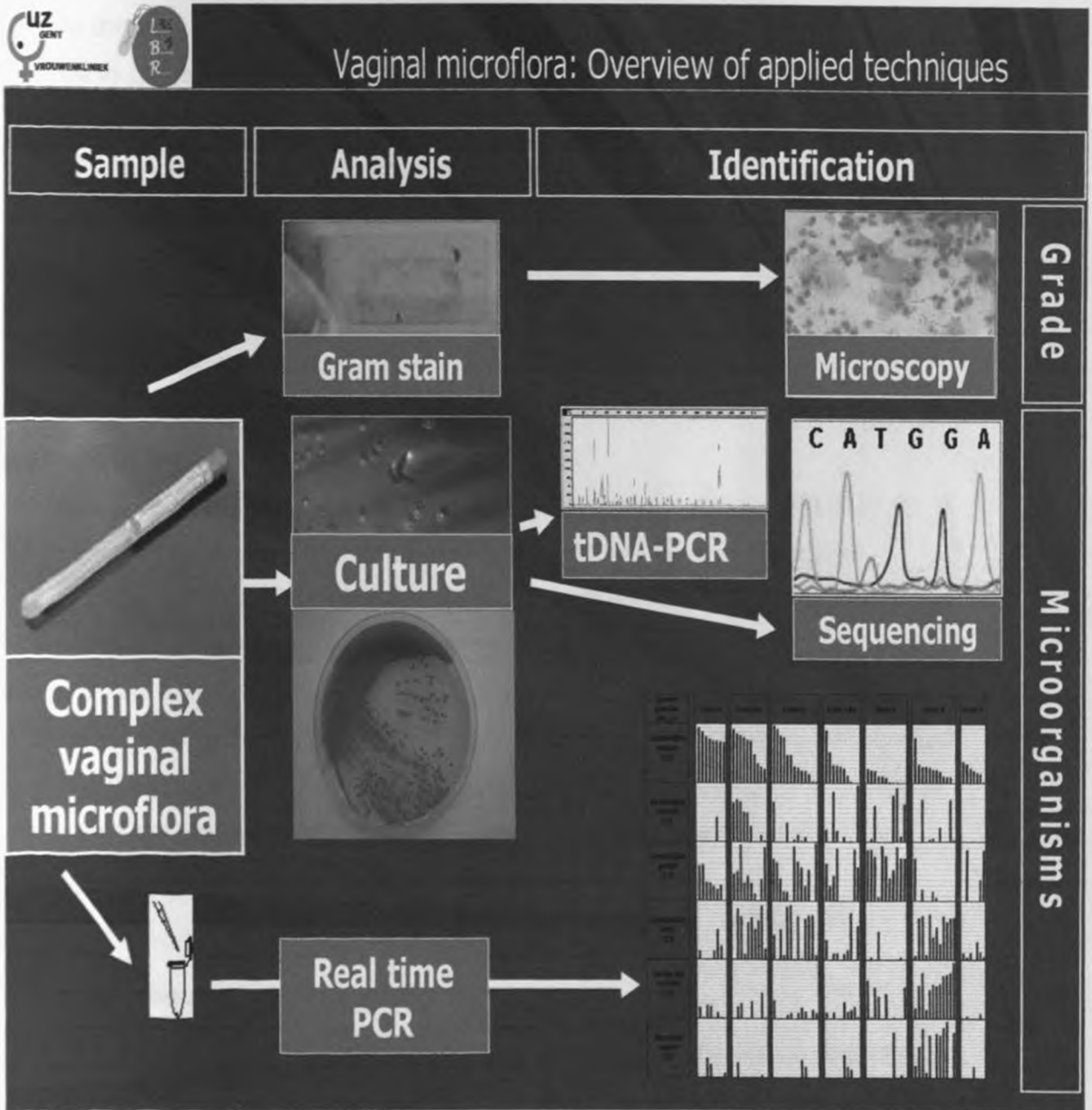
2. It was carefully removed to avoid contamination with the vulva and introitus microflora.

3. The swabs were transported to the laboratory within 4 hours of collection and processed immediately.

4. The 3 swabs were used as follows:

- ✓ Gram stain- prepared at clinical site.
- ✓ DNA isolation- put in dry transport tube and store at -80 °C.
- ✓ Culture- transported to the lab in transport medium.

Figure 17: General Scheme for the Analysis of Vaginal Microflora



3.4.2 SOP MAKING A GRAM STAIN

3.4.2.1 Background and Principle

The Gram stain is used to differentiate intact, morphologically similar bacteria into two groups based on cell color after staining. In addition, cell form, size and structural details are evident. Such preliminary information provides important clues to the type of organism(s) present and the nature of further techniques required to characterize them.

The Gram stain procedure (³⁸⁰Kruczak-Filipov and Shively, 1994) consists of:

- Staining a fixed smear with crystal violet.
- Applying iodine as a mordant.
- Decolorizing the primary stain with acetone.
- Counterstaining with safranin.

A crystal violet-iodine complex forms in the protoplast of all organisms stained by this procedure. Organisms able to retain this dye complex after decolorisation are classified as gram-positive.

The mechanism of the gram stain appears to be related to the presence of an intact cell wall able to act as barrier to decolorization of the primary stain.

Disruption or removal of the cell wall causes the protoplast of gram-positive cells to become decolorized and thus lose the gram-positive attribute.

Theory holds that during the Gram-stain procedure, the cell wall of gram-positive cells is dehydrated by the alcohol in the decolorizer and loses permeability, thus retaining the primary stain. The cell wall of the gram-negative bacteria cells has higher lipid content and becomes more permeable when treated with alcohol, resulting in loss of the primary stain.

3.4.2.2 Procedure

1. For Gram stain the swab was rolled several times onto the centre of a labelled, frosted slide, taking care not to spread the material over the slide.
2. It was air dried for a few seconds.

3. To ensure proper fixing of the material on the slide, the slide was fixed in methanol by flooding the slides with absolute methanol for 30-60 seconds and then air dry them.

4. Fixed slides were set in the slide box for transport to the lab for staining.

Reagents

1. Gram crystal violet (**Primary stain**)

2. Stabilized Gram Iodine (**Mordant**)

3. Gram decolorizer (**Decolorizer**)

4. Gram safranin (**Counter Stain**)

3.4.2.3 Staining Procedure

1. Flood the fixed smear with the primary stain (Gram Crystal Violet) and **stain for 1 min.**

2. Remove the primary stain by gently washing with **cold tap water (20 sec).**

3. Flood the slide with mordant (Stabilised Gram Iodine) (Lugol) and **retain on the slide for 1 min.**

4. Remove the mordant by gently **washing with tap water (20 sec).**

5. Decolorize (Gram decolorize) until solvent running from the slide is colorless (30-60 sec).

6. Wash the slide gently in cold tap water (20 sec).

7. Flood the slide with counterstain (Gram safranin) and stain for 30-60 sec.

8. Wash the slide with cold tap water (20 sec).

9. Blot with blotting paper or paper towel or allow to air dry.

10. Examine the smear under an oil immersion lens at a magnification of 1000x (100x eye piece and 10x lens).

11. The Gram-stain of the microflora will be assessed microscopically and graded according to the Nugent scoring criteria.

NB: This process can either be automated (MIRA STAINER, Merck) or done manually by **flooding slides with the dye above a drainage sink.**

3.4.2.4 Limitations of the Procedure

The Gram stain provides preliminary identification information only and is not a substitute for culture studies of the specimen. Gram stain results must be confirmed with additional procedures such as antigen tests and culture on media. Prior treatment with antibacterial drugs may cause gram-positive organisms from a specimen to appear gram-negative.

Gram stain results, including organism morphology, can be affected by the age of the isolate, cultures transferred from antibiotic-containing media as well as specimens collected from patients on antibiotics (Chapin, 1995).

Background material and artifacts can also interfere with interpretation.

3.4.2.5 Expected results and performance characteristics

(³⁸¹Chapin, 1995; ³⁸⁰Kruczak-Filipov and Shively, 1994; ³⁸²Magee *et al.*, 1975; ³⁸³Mangels *et al.*, 1984).

Reaction	4-Step Technique using Gram Safranin
Gram-positive	Purple-black cells
Gram-negative	Pink to red cells

3.4.3 SOP NUGENT SCORING CRITERIA

3.4.3.1 Background and Principle

When evaluating Gram stains for bacterial vaginosis, there are **3 different morphotypes to be evaluated:**

1. **Lactobacillus species**-large, sometimes long gram-positive rods
2. **Gardnerella vaginalis/ Bacteroides species**- small gram-variable/ gram-negative rods (these two morphotypes are combined due to their similar appearance on Gram stain).
3. **Mobiluncus** – curved gram-negative/ gram-variable rods.

Five oil immersion fields should be examined for the presence of *Lactobacillus*, *gardnerella vaginalis/ bacteroides* and *Mobiluncus*.

3.4.3.2 Procedure

Each should be evaluate using the following criteria:

0 per oil immersion field = 0

<1 per oil immersion field = 1+

1-4 per oil immersion field = 2+

5-30 per oil immersion field = 3+

> 30 per oil immersion field = 4+

MORPHOTYPE	EVALUATION	POINTS
LACTOBACILLUS	4+	0
	3+	1
	2+	2
	1+	3
	0	4
G .VAGINALIS/ BACTEROIDES	0	0
	1+	1
	2+	2
	3+	3
	4+	4
MOBILUNCUS	0	0
	1+/ 2+	1
	3+/ 4+	2

Figure 18-Formula for calculating by summing assigned points the Gram stain score (a score **0-3** is considered **negative** for BV; a score **4-6** is **Intermediate**; a score **7-10** is **positive** for BV according to the ¹⁵⁹Amsel *et al.*, 1983 and ⁷³Nugent *et al.*, 1991 criteria).

Gram stain results were reported as prescribed in Appendix 17.

NB: All slides were stored in slide boxes at room temperature for future reference and quality control at the department of Microbiology, Ghent University, Belgium.

3.4.3.3 Quality Control in grading the Microflora

In the routine microbiology laboratory at Ghent University, headed by Prof G. Claeys, the fixed smears were stained and examined under an oil immersion lens at a magnification of 1000x. Dr. Rita Verhelst counter-checked the slides. The results of the grading were recorded in the format shown in Appendix 18.

3.4.4 SOP BACTERIA CULTURE

3.4.4.1 Background and Principle

The GasPak EZ Gas Generating Sachet consists of a reagent sachet containing inorganic carbonate, activated carbon, ascorbic acid and water. When the sachet is removed from the outer wrapper, the sachet becomes activated by exposure to air. The activated reagent sachet and specimens are placed in the Gas Pak EZ Incubation Container and the container sealed. The sachet rapidly reduces the oxygen concentration within the container. At the same time, inorganic carbonate produces carbon dioxide.

For the cultivation of anaerobic bacteria, Gas Pak EZ Anaerobe Container System sachets produce an anerobic atmosphere within 2.5 h, with greater than 15% carbon dioxide within 24 h. This produces an atmosphere suitable to support the primary isolation and cultivation of anaerobic bacteria by use of gas generating sachets inside the incubation container.

3.4.4.2 Maintenance of GasPak EZ Incubation Container

1. Avoid contact with abrasives.
2. Avoid contact with solvents and detergents.
3. Rinse and dry thoroughly after cleaning with a mild detergent.
4. Never autoclave container or lid.
5. The GasPak rack plate holder is constructed of stainless steel and may be autoclaved.

3.4.4.3 Procedure

For all MIS study clients, culture of HVS was done at day 7 and 14 of each of the two menstrual cycles.

3.4.4.3.1 Preparation of Culture Medium

1. Weigh 42.5g of Columbia agar base.
2. Warm with agitation to dissolve the agar base in sterile distilled/ deionised water.
3. Autoclave at 121 °C for 15 min at 15 bars pressure.

4. Cool to 50-60 °C.
 5. Add 5 % sterile defibrinated sheep blood (50 mls blood per litre of medium) obtained from the Department of Clinical studies, College of Agriculture and Veterinary Sciences, University of Nairobi.
 6. Inside the laminar flow hood mix the medium well and pour 20 mls into each Petri dish without touching the sides.
 7. **Cover (partially) immediately** and leave the plates in the laminar flow hood to set.
 8. Store plates upside down at +2 °C to +8 °C and use within 4 weeks of preparation.
 9. Leave culture plates to equilibrate at room temperature for 10 min before the inoculation is done.
- NB: The culture and plates (Petri dishes) with medium [Columbia agar base supplemented with 5 % sheep blood] were prepared in the Microbiology Annex laboratory at College of Health Sciences, University of Nairobi.

3.4.4.3.2 Preparation of Bacteria Culture in the laboratory

1. Put the patient **specimen label on top and bottom** of the Petri dish. Place the label appropriately so as not to obstruct observation of culture in the lab.
2. Remove the HVS swab from the transport medium and inoculate the culture medium in the labeled plate.
3. Cover immediately to avoid contamination.
4. Incubate for 4 days under anaerobic conditions.

3.4.4.3.3 Procedure for Incubation in the Laboratory

1. In the bacteriology room inoculate the culture plates with the HVS brought from the clinical site in transport medium.
2. Transfer the inoculated culture plates to the rack of the culture jar.
3. Place the activated sachet of BD GasPak EZ Gas Generating Container Systems in the shielded clip on the plate rack with inoculated plates. **The sachet should be placed in the large shielded clip. Open the envelope to remove**

the reagent sachet and place the sachet behind the shielded clip of the rack with the printed side facing the inside of the jar (catalyst sachet toward the outside). Use one envelope for each GasPak-100 system.

4. Place the dry anaerobic indicator in the center of the metal indicator holder with the indicator pad upright. (Remove the number of indicator strips needed and close the bottle tightly. Do not touch indicator pad. Do not use indicator if white in color before use).

5. Place the rack in the GasPak jar.

6. Close the GasPak jar promptly and finger tighten only the GasPak-100 lid clamp. 7. Label the date that the culture will be processed, then place it inside the incubator at 37 °C.

8. Ascertain that the unit is working properly by observing the appearance of condensation on the inside of the jar. Periodically during incubation, observe that the indicator is decolorized.

9. When the incubation period is over, open the jar and allow it to aerate for approximately 15s prior to removing the rack and its contents so as to achieve atmosphere equilibration.

10. After 4 days of incubation all the isolates with different colony morphology will be selected for identification. Dispose of the GasPak EZ Container system sachets.

3.4.4.3.4 Results

Anaerobic conditions are achieved within 2.5 h, with greater than 15 % carbon dioxide within 24 h at 35 °C. Visible condensate should occur within 30 min of activation. Blood containing agar plates appear reduced within 2-4 h at 35 °C. The methylene blue BBL dry anaerobic indicator strip should appear reduced (white) within 2-4 h at 35 °C.

3.4.5 SOP ALKALINE DNA EXTRACTION

3.4.5.1 Background and Principle

After 4 days of anaerobic incubation all the isolates with different colony morphology were selected for identification. Bacterial DNA was extracted by simple alkaline lysis.

This protocol describes the extraction of DNA from bacteria by means of alkaline lysis. Alkaline Lysis Buffer (ALB) is a strong alkaline buffer consisting of a detergent, sodium dodecyl sulfate (SDS), and a strong base, sodium hydroxide (NaOH). SDS disrupts the cell membrane by denaturing the protein components and solubilizing the phospholipid component, leading to lysis and release of the cell contents (³⁸⁴Birnboim and Doly, 1979). SDS also dissociates nucleic acid-protein complexes. NaOH helps to break down the cell wall and disrupts the hydrogen bonding between the DNA bases, denaturing the DNA (³⁸⁵Birnboim, 1983). After incubation with ALB, the chromosomal DNA is released from the bacteria. It is a crude DNA preparation, useful for PCRs starting from cultured cells.

3.4.5.2 Procedure

1. Pick one pure colony using a sterile plastic loop and place in 20 µl SDS lysis buffer (0.25 % sodium dodecylsulfate-0.05 N NaOH) (Appendix 19).
2. Heat the sample at 95 °C for 15 min (**heating block, thermometer & timer**).
3. Spin briefly at maximum Minifuge speed (30 sec at 13000 g).
4. Dilute the sample (1:10) by addition of 180µl distilled water.
5. Spin briefly at maximum Minifuge speed (5 min 13000 g).
6. Store at -20 °C for at least 30 min.
7. The supernatant (Alkaline extract, AE) can be used for t-DNA PCR and capillary electrophoresis or stored frozen at -20 °C.

3.4.5.3 Preparation of Lysis buffer

1. Prepare 10% SDS.
2. Prepare 1 N NaOH (Merck).

3. Add 250 μ l 10% SDS, 500 μ l 1 N NaOH and 9.25 ml milliQ H₂O

= 10 mls 1X lysis buffer

4. Prepare 10 portions of 1X lysis buffer to give a total volume of 100 mls.

5. Filter-sterilize the lysis buffer with a 250 ml-0.22 μ m filter.

6. Aliquot into 1.7ml vials.

7. Store at room temperature.

NB: The colonies can be expanded to generate enough material. Use a sterile loop to streak the pure isolates on a plate. Pick up pure colonies (that have been cultured) with a cotton swab and put in TSB/ glycerol buffer for storage at -70 °C.

3.4.6 SOP EXTRACTION OF DNA

3.4.6.1 Background and Principle

Extraction of DNA was carried out on high vaginal swab (HVS) specimens previously stored frozen at -80 °C. Automated EasyMag extractor was used.

3.4.6.2 Procedure

The dry swab specimen from each patient was swirled for 15s in 1200 μ l of lysis buffer (20 mM Tris-HCl, pH 8.0; 2 mM EDTA; 1.2% Triton). To a 200 μ l aliquot was added 5 μ l 10% SDS to final concentration of 0.25%. Fifty units of mutanolysin (25 U/ μ l) (Sigma, Bornem, Belgium) were added and the samples incubated for 15 min at 37 °C. After the addition of 20 μ l Proteinase K (25 mg/ml) the samples were incubate for 15 minutes at 55 °C and vortexed after every 5 minutes. Nuclisens Easymag buffer (1800 μ l) (BioMérieux, France) was then added and samples incubate for 10 minutes at room temperature. Into Easymag disposable caps was added 2.0ml of the processed sample. To this was added 100 μ l mixture of magnetic silica and Easymag extraction buffer 3. The samples were then set inside the Easymag machine, and the extraction program started. The resulting DNA extract was used for real time bacteria specific PCR. DNA extracts were stored at -80 °C.

3.4.7 SOP TRANSFER DNA INTERGENIC SPACER LENGTH

POLYMORPHISM ANALYSIS (tDNA-PCR)

3.4.7.1 Background and Principle

Although tDNA-PCR can, in theory, be used directly on clinical samples for which only one or two species are expected to be present, isolation culture is usually needed to avoid interference of patterns from different strains rendering the obtained pattern uninterpretable. The same bacterial strain should be used as a reproducibility control with each PCR and capillary electrophoresis run.

This protocol was adapted from the method by ³⁵²Baele *et al.* (2000) (Appendix 20) and the starting material was the crude DNA preparation described in SOP 3.4.5.

Because capillary electrophoresis is used to separate the amplified tDNA-intergenic spacers, small PCR volumes, as low as 10 µL, are sufficient.

3.4.7.2 Procedure

3.4.7.2.1 Materials and Reagents

Bacterial cells

Formamide, deionized

Genomic DNA (up to 1 µg)

High Fidelity Mix (1.1X) (Invitrogen)

Oligonucleotides (Welsh & McClelland 1991):

primer 1 (T5A) 5'-AGTCCGGTGCTCTAACCAACTGAG-3' (10 µM)

primer 2 (T3B) 5'-GGTCGCGGGTTCGAATCC-3' (10 µM)

Label 1/5th of primer 2 with a fluorescent dye.

Size standards (labeled with a fluorescent dye) (Applied Biosystems or Beckman Coulter):

- GS-400 High Density size standard and GS-500 size standard (Applied Biosystems) (for use with the ABI Prism 310 Genetic Analyzer)

These standards contain ROX-labeled fragments in the range of 50 to 500 bp.

- LIZ600 size standard (Applied Biosystems) (for use with the ABI Prism 3100 or 3130XL Genetic Analyzers)

LIZ600 contains LIZ-labeled fragments in the range of 40 to 600 bp.

- CEQ-600 size standard (Beckman Coulter) (for use with the CEQ 8000 Genetic Analysis System)

Use with CEQ sample loading solution (SLS) (Beckman Coulter 608082).

3.4.7.2.2 Equipment

Capillary electrophoresis apparatus (e.g., ABI-Prism 310, 3100 or 3130XL

Genetic Analyzer; Applied Biosystems or CEQ 8000; Beckman Coulter)

Heater

Microcentrifuge

Plates, microtiter

Thermal cycler

3.4.7.2.3 Software for data analysis

- Basehopper (www.BaseHopper.be)
- Genescan Analysis software (Applied Biosystems) (for use with ABI Prism only)
- Phylip/Neighbor (<http://evolution.genetics.washington.edu/phylip.html>)

A manual is downloadable from the same website.

- TreeView (<http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>)

3.4.7.3 tDNA-PCR

Prepare the PCR using a 10- μ L total volume per sample:

9.1 μ L High Fidelity Mix 1.1X

0.1 μ L primer 1

0.1 μ L primer 2 (of which 0.02 μ L is fluorescently labeled)

0.7 μ L DNA template from SOP 5.5

Perform the following cycling program:

No. of Cycles	Temperature	Time
1	94 °C	2 minutes
30	94 °C	30 seconds
	50 °C	1 minute
	72 °C	1 minute

1	72 °C	30 minutes
1	10 °C	hold

3.4.7.4 Capillary Electrophoresis

3.4.7.4.1 Prepare samples for capillary electrophoresis on an ABI Prism 310

- i. For each PCR product, mix 12 μL of deionized formamide with 0.3 μL of GS-400 High Density size standard and 0.2 μL of GS-500 size standard (which both have ROX-labeled fragments in the range of 50 to 500 bp for use with the ABI Prism 310). Prepare the mixture of formamide and marker in a total volume sufficient for all PCR products, and divide into 12.5 μL /well in microtiter plates.
- ii. Add 1 μL of tDNA-PCR product from Step 3.4.7.3 to each well. Denature the mixtures by heating at 95 °C for 3 minutes. Place the mixtures directly on ice for at least 15 minutes.

3.4.7.4.2 Perform capillary electrophoresis:

- i. Use the following conditions for electrophoresis using an ABI Prism 310:

Temperature	60 °C
Constant voltage	1.5 kV
Current: approximately	10 mA
Capillary length	47 cm (50- μm diameter)
Performance Optimized Polymer	4

3.4.7.5 Data Analysis

To analyze data generated using an ABI system:

- i. Normalize the electropherograms using GeneScan Analysis software.

The fragment lengths are derived from the peak positions after interpolation with the peak positions of the size standard fragments.

- ii. In GeneScan Analysis, select the sample files for identification. Extract the sample files in table format using the "export tables" menu. Save resulting data as a file on your computer, preferably in C:\Samples or C:\Tables.

The digitized fingerprints (sample files) can then be given species names and strain designations.

13. Using Basehopper, compare the sample files to an existing library (database) of other sample files using the Dice, Jaccard or differential base pairs (dbp)

algorithm. To generate a library with known fingerprints (libraries), use the BaseHopper manual.

For tDNA-PCR, the dbp algorithm has been shown most useful, because additional peaks that are not reproducible are not taken into account in the similarity calculation, and thus do not influence the similarity percentage between an unknown strain and the library pattern.

For cluster analysis using Basehopper, calculate the distance matrix using the Dice, Jaccard or differential base pairs (dbp) algorithms. Cluster the samples using Phylip/Neighbor, employing the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) or Neighbor Joining algorithm.

Construct a dendrogram using TreeView, which can be opened directly through the BaseHopper menu.

Optional: Represent the fingerprints graphically using the Graph option of BaseHopper.

3.4.7.6 Troubleshooting

3.4.7.6.1 Problem 1: There is amplification in the negative PCR control.

Solution: Due to the manipulation of PCR products in all PCR-based methods, the reactions can become contaminated. If negative controls produce a DNA smear, several steps can be taken to eliminate the contamination:

- Repeat the PCR using fresh reagents.
- Use filtered tips to avoid introduction of contaminants via aerosol from the pipette.
- Physically separate the areas in the laboratory where reactions are set up and processed. Prepare and pipette the PCR mixture at one bench, and then add the DNA to the reaction in a different location in the laboratory. Also, use different pipettes for reaction preparation and pipetting of DNA.
- It is possible to prepare the tDNA-PCR reaction mixture in a large volume and to aliquot large series of 9.3- μ L volumes into tubes or microtiter plates. These can be stored at -80°C . Only the DNA extract must be

added when an isolate is ready to be identified. This may reduce the chance of contamination by reducing the number of manipulations.

Note that with techniques like tDNA-PCR, which start from a high concentration of target DNA, contamination is a minor problem. Even when negative controls show some amplification, the contaminating fragments are not observed after amplification of the bacterial DNAs, probably due to competition in favor of the abundantly present target DNA.

3.4.7.6.2 Problem 2: No amplification is observed.

Solution: Check the quality of the starting DNA. Perform an alternative DNA extraction method if necessary.

The species of a limited number of groups (e.g., corynebacteria) often yield no or a low number of amplicons. tDNA-PCR cannot be used for most species of these groups using the described primer pair. Select other tRNA primers.

3.4.7.6.3 Problem 3: The obtained peak pattern is not present in the database.

Solution: Mixed cultures may be present in the DNA sample. Repeat isolation culture of the sample. Alternatively, the species may not yet be in the identification library. Identify the strain by using a reference technique, e.g., 16S rRNA gene sequencing. Add the name of the species and its corresponding fingerprint to the library.

3.4.8 SOP REAL TIME PCR

3.4.8.1 Background and Principle

This protocol describes the procedure of a real-time PCR on the ABI 7000. With real-time PCR, or quantitative PCR (qPCR), it is possible to follow the amplification of a DNA fragment or region. The detection is based on the SYBR Green 1 molecule. This molecule is incorporated in the minor groove of double stranded DNA (dsDNA). The resulting DNA-SYBR Green 1-complex emits green light ($\lambda_{\max} = 522\text{nm}$) which is detected by the ABI 7000. The intensity of this emission signal is proportional to the amount of dsDNA present in the reaction.

Therefore, at each step of the PCR reaction, the signal intensity increases as the amount of double stranded product increases. This provides a very simple and reliable method to monitor PCR reactions in real time and to quantify the amount of initial target DNA present.

3.4.8.1 Procedure

The qPCR Core Kit for SYBR Green I (Eurogentec) was applied and analysis was performed on the ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA). Reactions were done in PCR mixtures containing 2.5 µl of DNA extract, 2.5 µl of 10x Reaction Buffer, 3.5 mM MgCl₂, 0.2 mM dNTP mixture, 0.625 U HotGoldStar *Taq* polymerase, 0.75 µl SYBR[®] Green I, diluted 10-fold in DMSO and the appropriate primer concentration, adjusted with HPLC grade water to 25 µl. Each run included a standard curve and each sample was run in triplicate. In case the result was not in the range of the standard curve, the samples were diluted tenfold and analyzed in triplicate again. The median log₁₀ cells/ml were expressed as per 1 ml elution buffer.

For the MIS study real-time PCR was performed on part of the specimens due to cost considerations. Three *Lactobacillus* species (*L. crispatus*, *L. iners* and *L. jensenii*) and two species associated with bacterial vaginosis, *Atopobium vaginae* and *Gardnerella vaginalis*, were quantified.

3.4.8.1.1 Equipment

- 96- Well Base Micro Amp[®]
- Adhesive Film Applicator Micro Amp[®]
- Optical Film Compressor Pad Micro Amp[®]
- Microcentrifuge VWR Galaxy™ Minstar.
- ABI PRISM 7000 Sequence Detection System
- Plate Centrifuge
- Pipette 0.5 µl-10µl
- Pipette 5µl-50µl

Pipette 40µl-200µl

Pipette 200µl-1000µl

Automatic pipette

3.4.8.1.2 Disposables

Optical 96-Well Reaction Plate MicroAmp®

Optical Adhesive Film Micro Amp®

Excel Worksheet Real-time PCR

1.5ml Eppendorf tube, 2.0 ml vial tube or 14ml Falcon tube (See: Preparation of the mastermix: 6)

Filter tips 1000µl

Filter tips 200 µl

Filter tips 100 µl

Filter tips 10 µl

Mini-Tork paper roll

3.4.8.1.3 In Advance

1. On the ABI 7000 machine open the Excel sheet 'form_ PCR_Realtime' and select the strain.

2. Fill in the number of tubes and press 'enter'.

For calculating the number of tubes, take into account:

- Number of samples x 3 (working in triplicate) or x2 (working in duplicate)
- Standard series x3 (working in triplicate) or x2 (working in duplicate)
- 2 negative controls.

3. Print the sheet.

4. Take ice in a box.

5. Keep the qPCR Core Kit and primers on ice.

6. Thaw the DNA extracts in post-PCR room.

3.4.8.1.4 Preparation of the mastermix

All procedures are carried out in PCR room.

1. Put on the blue lab coat in the lock.
2. Wear gloves.
3. Clean the bench with ethanol.
4. Put a piece of paper roll on the bench.
Use this to put your used filter tips on.
5. Short spin the tubes with reagents for the mastermix in the centrifuge.
6. Make PCR –Mix with the volumes as calculated on the worksheet: start with the highest amount (HPLC water).

Depending on the total number of tubes, make the mastermix in:

- A 1.5 ml Eppendorf tube : 1 to 60 tubes
- A 2.0 ml vial : 61 to 80 tubes
- A 14ml Falcon : 81 to 96 tubes

7. Short-spin the mastermix.
8. Take a 96- Well Optical Reaction Plate and place it on a 96-well base.
Make sure the optical plate does not become dirty.
9. Divide the mastermix in the wells using the automatic pipette (see: Protocol Automatic Pipette LBR-T001).
Put the automatic pipette back in the charger after use.
10. Cover the plate with adhesive film without removing the film cover.
Do not stick the adhesive film on the plate, this step is only to avoid contamination during the transport to room 305, where the film will be ticked on the plate after adding the samples.
11. Clean the bench with ethanol.
12. Put the piece of paper roll with the used tips and gloves in the dustbin.
13. Take off the blue lab coat in the lock.

3.4.8.1.5 Adding DNA-Extracts to the mastermix

In sample room:

1. Put on the blue lab coat in the lock.
2. Put on the gloves.

3. Clean the bench with ethanol.
4. Put the piece of paper roll on the bench.
Use this to put your used filter tips on.
5. Pippet the volume of sample or standard in the wells, as noted on the Excel sheet.
To optimize detection, avoid having two of the same samples or the same standards in the same row.
6. Close the 96-well optical plate with optical adhesive film applicator.
7. Clean the bench with ethanol.
8. Put the piece of paper roll with the used tips and gloves in the dustbin.
9. Take off coat.
10. Centrifuge the plate for 45 seconds to remove air bubbles using the plate centrifuge.
11. Check that air bubbles are absent. If not, repeat step 9.

3.4.8.1.6 Starting the real –time PCR on the ABI 7000.

Starting the ABI 7000 and ABI PRISM software.

1. Start up the ABI 7000 by pushing the button on the right hand corner
2. Start up the computer.
3. Double click on the 'ABI Prism 7000' icon on the desktop of the PC.
4. Select 'file' > 'new' and click 'ok' to accept the standard set up parameters for the 96-well plate.

A plate-document opens and the connection with the ABI PRISM ids established. In the right hand corner of the window you should see 'connected'.

3.4.8.1.7 Selecting the detector and defining the wells.

1. Click on 'view' > 'well inspector'.
2. Click on the 'add detector' and select the detector to be used.
3. Click on the 'add to plate document' button.
4. Click on 'done'.

5. Select the wells with samples, standards and controls in 'Plate /Setup' by clicking and dragging.
6. Assign the selected detector to the selected wells by clicking on 'use'.
7. Select the wells with samples by clicking and indicate them as 'unknown' by clicking on 'task' and selecting 'unknown'.
8. Select wells with standards by clicking and indicate them as 'standard' by clicking on 'task' and selecting 'standard'.
9. Select the wells with negative controls and indicate them as 'negative control' by clicking on the 'task' and selecting 'NTC'.
10. Close the 'well inspector' dialogue window by clicking 'done'
11. Save the plate document by selecting 'file'> 'save as' and giving the file a name.
12. Select SDS document (*.sds) in the menu 'save as type'

3.4.8.1.8 Setting the PCR cycle.

1. Click on the 'instrument' tab in the plate document.
A cycle appears
2. Adapt the cycle according to the cycle described on the real-time PCR sheet.
If an additional step is required, click between two steps.
If a temperature has to be changed, click in the rectangle and change it.
If a time has to be changed, click in the rectangle and change it.
3. Select 'dissociation step'.
4. Adjust sample volume to 25µl.

3.4.8.1.9 Starting the real-time PCR.

1. Push the button on the block so that the sample tray moves forward.
2. Put the 96-well plate on the sample block with A1 in the upper left corner.

3. Place the compression pad on the 96-well plate with the blinking gray side up.
4. Push gently against the tray. The tray will automatically move to its position.
5. Push lever down to close the ABI 7000.
6. Click 'start' on the PC.
7. When the real-time PCR is finished 'idle' appears.

3.4.8.1.10 Quality Control Assays

1. The study specimens were transported to Belgium by courier, courtesy of Prof. M. Temmerman, Prof. Mario Vaneechoutte and VLIR.
2. All Gram-stained slides were stored in slide box at room temperature for future reference and quality control at the department of Microbiology, University of Ghent, Belgium, courtesy of Dr. Rita Verhelst and Prof. G. Claeys.
3. Quality control for microbiology and DNA isolations were carried out in Belgium, Department of Microbiology, University of Ghent, courtesy of Prof. M. Vaneechoutte.

3.5 STATISTICAL ANALYSIS

Prevalence rates were compared between groups through Chi square test, or Fischer's Exact Test when appropriate. Statistical significance was accepted at the significance level $\alpha = 0.05$. All analyses were performed with statistical software package PASW v18.0 (Chicago, Illinois), courtesy of Dr. H. Verstraelen. Real time PCR plots were constructed on SPSS version 13.0.

CHAPTER FOUR

4.1 RESULTS

4.1.1 Cohort characteristics

Baseline demographic characteristics of 41 HIV+ve and 33 HIV-ve women are presented in Table 4.1. All the participants were sufficiently literate to follow the appointment schedule for return visits to the MIS study.

For most parameters taken at enrolment, no significant differences existed between the two groups. As expected, the HIV+ve women had lower CD₄ counts ($p < 0.001$) and condom use was more common among them (61%) compared to the HIV-ve women (18.2%) ($p < 0.001$). Another difference was antibiotic prophylaxis among the HIV+ve group (78%, $p < 0.001$), because the Kenya national guidelines for treatment and care for HIV-infected persons allow continuous antibiotic intake for prevention of recurrent bacterial infections. About 63.4% of the HIV+ve participants were married compared to the 39.4% HIV-ve ones ($p = 0.001$). The HIV+ve women had significantly lower levels of schooling ($p = 0.005$).

Table 4.1 Descriptive characteristics of the study population at enrolment, expressed as percentages.

Parameter	Categories	HIV+ve (41)	HIV-ve (33)	<i>p</i> value
Age (years)	21-28	14.6	24.2	0.4
	29-36	53.7	39.4	< 0.001
	37-44	31.7	36.4	
CD ₄ count (cells/ μ L)	250-500	56.1	3.0	< 0.001
	501-750	31.7	15.2	
	751-1000	7.3	54.5	
	1001-1500	4.9	27.3	
Vaginal pH	3.0-4.5	73.2	63.6	0.4
	> 4.5	26.8	36.4	
<i>Candida</i>	Positive	36.6	27.3	0.4
	Negative	63.4	72.7	

Gram stain	Grade 0	9.8	6.1	0.7
	Grade I	56.1	72.7	
	Grade II	9.8	6.1	
	Grade III	22.0	12.2	
	No score	2.4	3.0	
Antibiotic prophylaxis	Yes	78.0	0.0	<0.001
	No	22.0	100.00	
Height (cm)	130-144	2.4	0.0	0.4
	145-159	46.3	30.3	
	160-174	48.8	66.7	
	> 175	2.4	3.0	
Body weight (kg)	45-54	26.9	18.2	0.3
	55-64	26.9	36.4	
	65-74	31.7	18.2	
	75-84	12.2	15.2	
	85-94	0	9.1	
	> 95	2.4	3.0	
Age at first sex (years)	14-15	9.8	6.1	0.7
	16-18	43.9	36.4	
	19-23	31.7	33.3	
	24-28	12.2	18.2	
	> 29	0	3.0	
	N/A	2.4	3.0	
Lifetime partners	0-1	19.5	27.3	0.3
	2-3	48.8	42.4	
	4-7	24.4	30.3	
	8-10	7.3	0.0	
Current partners	0	24.4	30.3	0.6
	1	75.6	69.7	
Marital status	Married	63.4	39.4	0.001
	Single/Separated	22.0	60.6	
	Widow	14.6	0	
Condom use	Yes	61.0	18.2	<0.001
	No	39.0	81.8	
Level of schooling	Primary and below	46.3	12.2	0.005
	Secondary	51.2	78.8	
	Tertiary	2.4	9.1	
Occupation	Housewife	36.6	30.3	0.2
	Farmer	14.6	6.10	
	Business	24.4	15.2	
	Formal employment	24.4	42.4	
	Student	0	6.10	

4.1.2 Fluctuation of the Gram stain score

Bacterial vaginosis was diagnosed in 17.5% of enrolment visits. Table 4.2 shows the fluctuation in Gram stain score over two menstrual cycles. Approximately, 42.4% of HIV-ve and 31.7% of HIV+ve women harboured a normal vaginal microflora on all six visits. While BV was virtually absent in HIV-ve women using condoms, half of the HIV+ve women using condoms had a disturbed microflora on three or more visits. Of all women not using condoms, 41% had BV on at least half of the visits. The microflora of respectively 11.1% and 14.6% of HIV-ve and HIV+ve women was disturbed on all follow up visits.

As shown in table 4.3, the number of grade I, II and III Gram stains was similar throughout the phases of the menstrual cycle in both study groups.

	(17)	(24)
Normal	42.4	31.7
Grade I	11.1	16.7
Grade II	14.6	20.8
Grade III	23.2	29.2
Intermediate or BV	11.1	17.0

Table 4.2 Percentages of the fluctuation of the Gram stain scores of HIV-ve and HIV+ve women taken at six visits each, spanning two menstrual cycles.

	HIV-ve		HIV+ve	
	No	Yes	No	Yes
	Condom Use			
Total Number	(27)	(6)	(17)	(24)
Invariably normal	33.3	83.3	47.1	20.8
Single intermediate or BV episode	11.1	16.7	11.8	29.2
Two intermediate or BV episodes	14.8	0	5.8	0
Three or more intermediate or BV episodes	29.6	0	17.6	37.5
Invariably intermediate or BV episode	11.1	0	17.6	12.5

Table 4.3 Distribution of Nugent scored vaginal microflora grades among HIV+ve and HIV-ve women during different phases of two menstrual cycles. Percentages in parentheses.

Grades	HIV+ve (n = 41)			HIV-ve (n = 33)		
	Follicular	Ovulation	Luteal	Follicular	Ovulation	Luteal
0	2 (2.4)	4 (4.9)	0 (0.0)	1 (1.5)	1 (1.5)	0 (0.0)
I	42 (51.2)	49 (59.8)	53 (64.6)	41 (62.1)	45 (68.2)	44 (66.7)
II	18 (22.0)	12 (14.6)	15 (18.3)	11 (16.7)	8 (12.1)	9 (13.6)
III	17 (20.7)	16 (19.5)	14 (17.1)	11 (16.7)	12 (18.2)	11 (16.7)
Missing	3 (3.7)	1 (1.2)	0 (0.0)	2 (3.0)	0 (0.0)	2 (3.0)
Total	82	82	82	66	66	66

4.1.3 Presence of bacteria species according to HIV status

The species cultured and identified in the HIV+ve and HIV-ve women in this study are presented in table 4.4 and figure 19. A total of 1,020 isolates were cultured. Of these, about 26% remained unidentified since no corresponding t-DNA PCR fingerprint could be found in the existing database or because no amplification was obtained. A total of 51 species were identified, of which 9 belonged to the genus *Lactobacillus*: *L. coleohominis*, *L. crispatus*, *L. gasseri*, *L. iners*, *L. jensenii*, *L. mucosae*, *L. reuteri*, *L. salivarius*, *L. vaginalis*. About 54.5% of HIV-ve and 60.9% of HIV+ve women were colonized by lactobacilli on at least one visit. Only 9.1% and 29.3% HIV-ve and HIV+ve women respectively had lactobacilli on at least three visits. The most common *Lactobacillus* species recovered were *L. crispatus*, *L. iners*, *L. jensenii* and *L. vaginalis*. *Lactobacillus crispatus* was cultured on three out of four visits for two women, it occurred twice in five women and only once in 11 women. *Lactobacillus iners* was cultured on three visits for two women, it was found twice in two women and only once in 13 women. *Lactobacillus jensenii* was recovered on three out of four visits in five women, on two visits in two women and on one visit for four women. Two species occurred more frequently in the HIV+ve women compared to those HIV-ve: *L. iners* ($p=0.02$) and *L. jensenii* ($p=0.01$). *Lactobacillus coleohominis* and *L. reuteri* were not detected in the HIV+ve women, whereas *L. mucosae* and *L. salivarius* were absent in the HIV-ve women.

Gardnerella vaginalis and *Peptoniphilus lacrimalis* occurred more frequently in HIV+ve women ($p=0.01$), whereas *Dialister micraerophilus* was more common in the HIV-ve group ($p=0.02$) as was *Streptococcus agalactiae* ($p=0.04$).

The presence of most species did not differ according to condom use.

Lactobacillus spp. was cultured from 52.0% of women using condoms and from 66.6% of women not using condoms. In 33.3% of all women using condoms, lactobacilli were cultured on three out of four visits, compared to 3.7% of HIV-ve women and 11.7% of HIV+ve women not using condoms (Table 4.5a). The presence of *L. crispatus* and *L. jensenii* differed according to condom use (Table

4.5b). While these species were more frequently cultured from condom users among HIV-ve women (20.8% vs. 10.2%), their presence was higher in the HIV+ve women not using condoms (26.5% vs 16.7%). *L. iners* was the only *Lactobacillus* species that was cultured more frequently in the HIV+ve group using condoms. *S. agalactiae* carriage was high in women using condoms. Respectively, 66.6% of HIV-ve and 21.7% of HIV+ve women using condoms carried this species compared to 14.8% and 5.8% in the non-condom using groups. Bifidobacteria were not cultured in this cohort despite being visible on gram-stained vaginal smears.

Table 4.4 Percentage of 250 visits during which 44 HIV+ve and 33 HIV-ve women had a positive culture.

Species	HIV pos women ^a	HIV neg women ^b	P value
<i>Acinetobacter haemolyticus</i>	1.4	0	0.2
<i>Acinetobacter lwoffii</i>	0.7	0	0.3
<i>Anaerococcus prevotii</i>	2.1	4.6	0.2
<i>Anaerococcus tetradius</i>	0.7	0	0.3
<i>Anaerococcus vaginalis</i>	0.7	0.9	0.4
<i>Atopobium vaginae</i>	0	0.9	0.2
<i>Bacteroides coagulans</i>	0.7	0	0.3
<i>Bacteroides ureolyticus</i>	0.7	0.9	0.4
<i>Clostridia bacterium</i>	0.7	0	0.3
<i>Dialister micraerophilus</i>	0	3.7	0.02
<i>Enterococcus faecalis</i>	7.7	14.8	0.07
<i>Escherichia coli</i>	9.2	10.2	0.7
<i>Fingoldia magna</i>	16.2	22.2	0.2
<i>Gardnerella vaginalis</i>	12	3.7	0.01
<i>Klebsiella pneumoniae</i>	0.7	0	0.3
<i>Lactobacillus coleohominis</i>	0	1.9	0.09
<i>Lactobacillus crispatus</i>	11.3	14.8	0.4
<i>Lactobacillus gasseri</i>	1.4	1.9	0.7
<i>Lactobacillus iners</i>	15.5	6.5	0.02
<i>Lactobacillus jensenii</i>	13.4	4.6	0.01
<i>Lactobacillus mucosae</i>	0.7	0	0.3
<i>Lactobacillus reuteri</i>	0	1.9	0.09
<i>Lactobacillus salivarius</i>	2.8	0	0.07

<i>Lactobacillus vaginalis</i>	7.7	4.6	0.3
<i>Mobiluncus curtisii</i>	0.7	0	0.3
<i>Peptoniphilus asaccarolyticus</i>	27.5	29.6	0.7
<i>Peptoniphilus lacrimalis</i>	7	0.9	0.01
<i>Peptoniphilus sp.</i>	0.7	2.8	0.1
<i>Peptostreptococcus anaerobius</i>	13.4	14.8	0.7
<i>Peptostreptococcus hydrogenalis</i>	0.7	0	0.3
<i>Peptostreptococcus indolicus</i>	0	0.9	0.2
<i>Porphyromonas somerae</i>	0.7	1.9	0.3
<i>Porphyromonas sp.</i>	0	0.9	0.2
<i>Prevotella bivia</i>	12.7	13	0.9
<i>Prevotella buccalis</i>	3.5	0.9	0.1
<i>Prevotella corporis</i>	0.7	2.8	0.1
<i>Prevotella disiens</i>	2.1	0	0.1
<i>Prevotella timonensis</i>	0.7	1.9	0.3
<i>Propionibacterium acnes</i>	0.7	0	0.3
<i>Pseudomonas mendocina</i>	0	0.9	0.2
<i>Staphylococcus aureus</i>	0.7	1.9	0.3
<i>Staphylococcus epidermidis</i>	17.6	19.4	0.7
<i>Staphylococcus hominis</i>	1.4	0	0.2
<i>Streptococcus agalactiae</i>	4.9	12	0.04
<i>Streptococcus anginosus</i>	9.9	9.3	0.8
<i>Streptococcus mitis</i>	2.1	3.7	0.4
<i>Streptococcus salivarius</i>	0.7	0	0.3
<i>Streptococcus sp.</i>	0.7	0	0.3
<i>Ureaplasma parvum</i>	0.7	0	0.3
<i>Veillonella atypica</i>	5.6	11.1	0.1
<i>Veillonella parvula</i>	0.7	0	0.3

Legend:

^a 142 visits

^b 108 visits

NB: Statistically significant p-values ($p < 0.05$) are indicated in boldface type.

Figure 19- Bacterial species present in more than 2% of the 74 women studied.

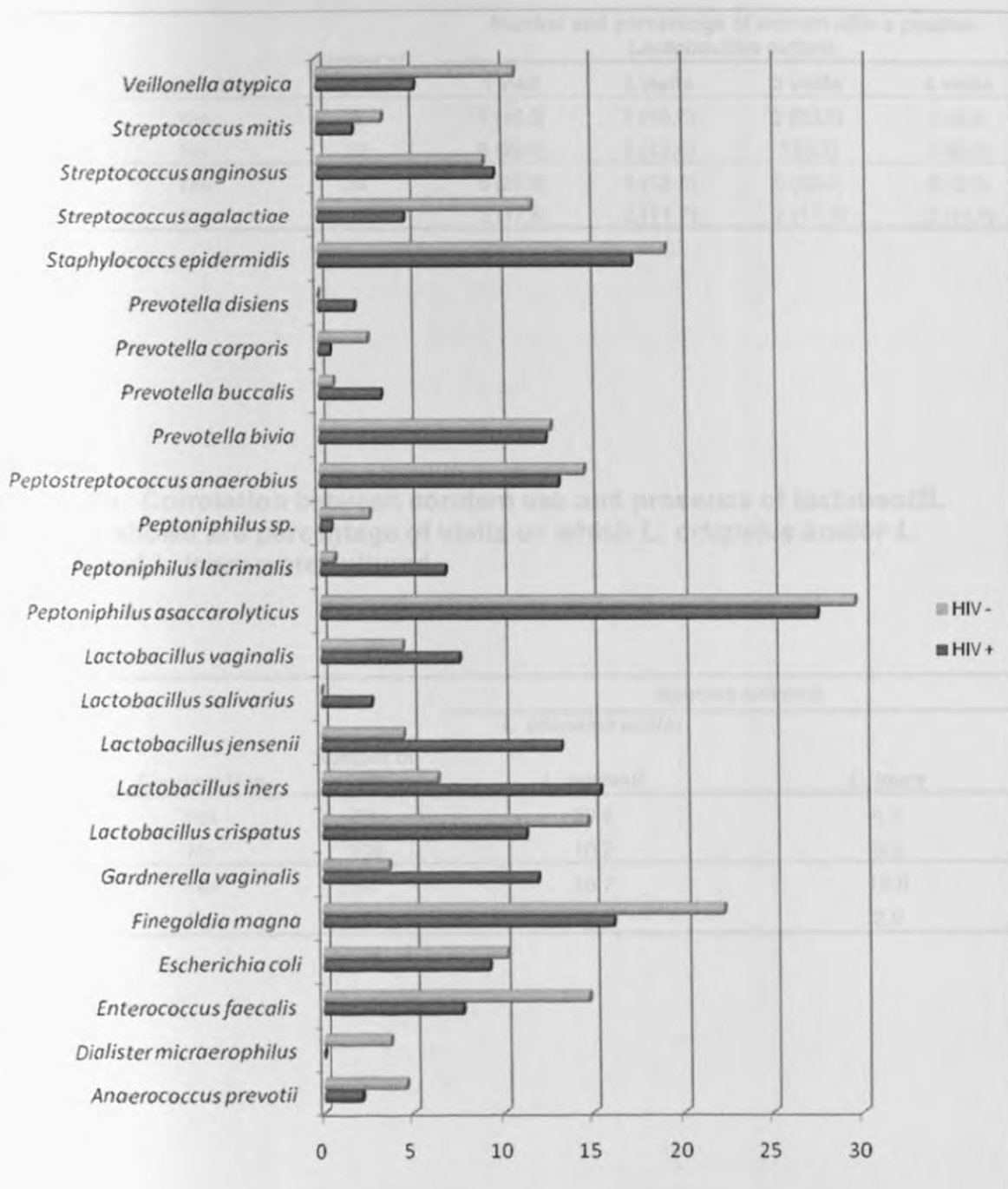


Table 4.5a Correlation between HIV status, condom use and the presence of *Lactobacillus* in culture.

HIV status	Condom Use	Number of women	Number and percentage of women with a positive <i>Lactobacillus</i> culture			
			1 visit	2 visits	3 visits	4 visits
Negative	Yes	6	1 (16.6)	1 (16.6)	2 (33.3)	0 (0.0)
	No	27	8 (29.6)	5 (18.5)	1 (3.7)	0 (0.0)
Positive	Yes	24	5 (20.8)	3 (12.5)	8 (33.3)	0 (0.0)
	No	17	3 (17.6)	2 (11.7)	2 (11.7)	2 (11.7)

Table 4.5b Correlation between condom use and presence of lactobacilli. Numbers shown are percentage of visits on which *L. crispatus* and/or *L. jensenii* and *L. iners* were cultured

HIV status	Condom Use	Number of visits	Species cultured	
			<i>L. jensenii</i>	<i>L. iners</i>
Negative	Yes	24	20.8	4.2
	No	108	10.2	5.5
Positive	Yes	96	16.7	15.0
	No	68	26.5	2.9

4.1.4 Real Time PCR Analysis based on Gram Stain Category

Figure 20 shows the relative concentrations by real time PCR of *L. crispatus*, *L. jensenii*, *L. iners*, *G. vaginalis* and *A. vaginae* as analysed based on gram stain result. Results show a high load of *L. iners* in the normal VMF and which increased during BV. There were high levels of *L. jensenii* in half of the women with BV while *L. crispatus* was absent in BV cases. *G. vaginalis* concentration increased progressively from normal to BV microflora while *A. vaginae* was absent in normal microflora but was detectable in intermediate gram stain and increased progressively during the BV phase.

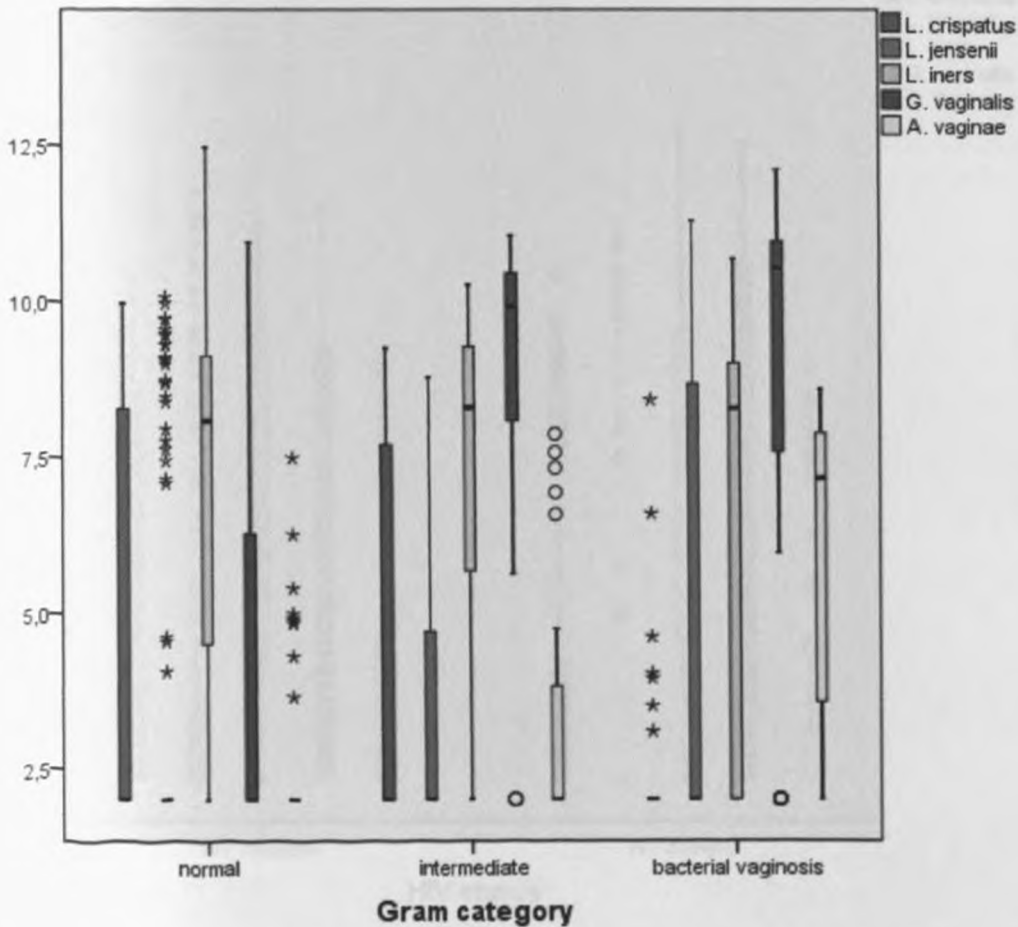


Figure 20- Relative concentrations of *L. crispatus*, *L. jensenii*, *L. iners*, *G. vaginalis* and *A. vaginae* based on gram stain result and detected by real time PCR.

4.1.5 Real Time PCR Analysis based on HIV Status

Figure 21 shows the relative concentrations by real time PCR of *L. crispatus*, *L. jensenii*, *L. iners*, *G. vaginalis* and *A. vaginae* as analysed based on HIV status. There was high concentration of *L. iners* and *G. vaginalis* in both the HIV+ve and HIV-ve groups. Although condom use was significantly higher in HIV+ve women, the number of women harboring the protective *L. crispatus* and the concentration of *L. crispatus* was remarkably lower compared to HIV-ve women. *A. vaginae* was present in both groups but *L. jensenii* was not detectable in the HIV+ve women.

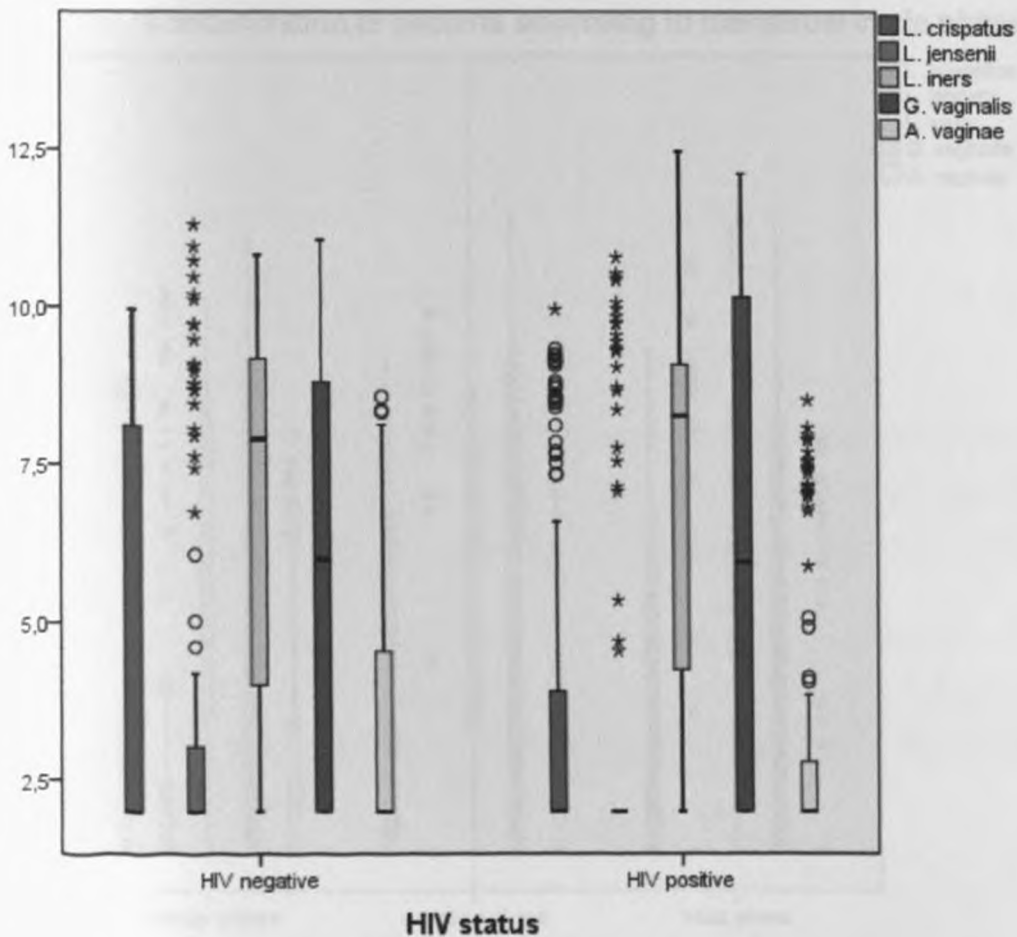


Figure 21- Relative concentrations of *L. crispatus*, *L. jensenii*, *L. iners*, *G. vaginalis* and *A. vaginae* based on HIV status and detected by real time PCR.

4.1.6 Real Time PCR Analysis based on phase of menstrual cycle

Figure 22 shows the relative concentrations by real time PCR of *L. crispatus*, *L. jensenii*, *L. iners*, *G. vaginalis* and *A. vaginae* as analysed based on phase of menstrual cycle. There was high concentration of *L. iners* and *G. vaginalis* throughout the menstrual cycle. Concentration of *L. crispatus* increased while concentration of *A. vaginae* decreased throughout the menstrual cycle. *L. jensenii* was not detectable after the initial phase of the menstrual cycle where it was comparatively low.

Concentration of bacteria according to menstrual cycle phase

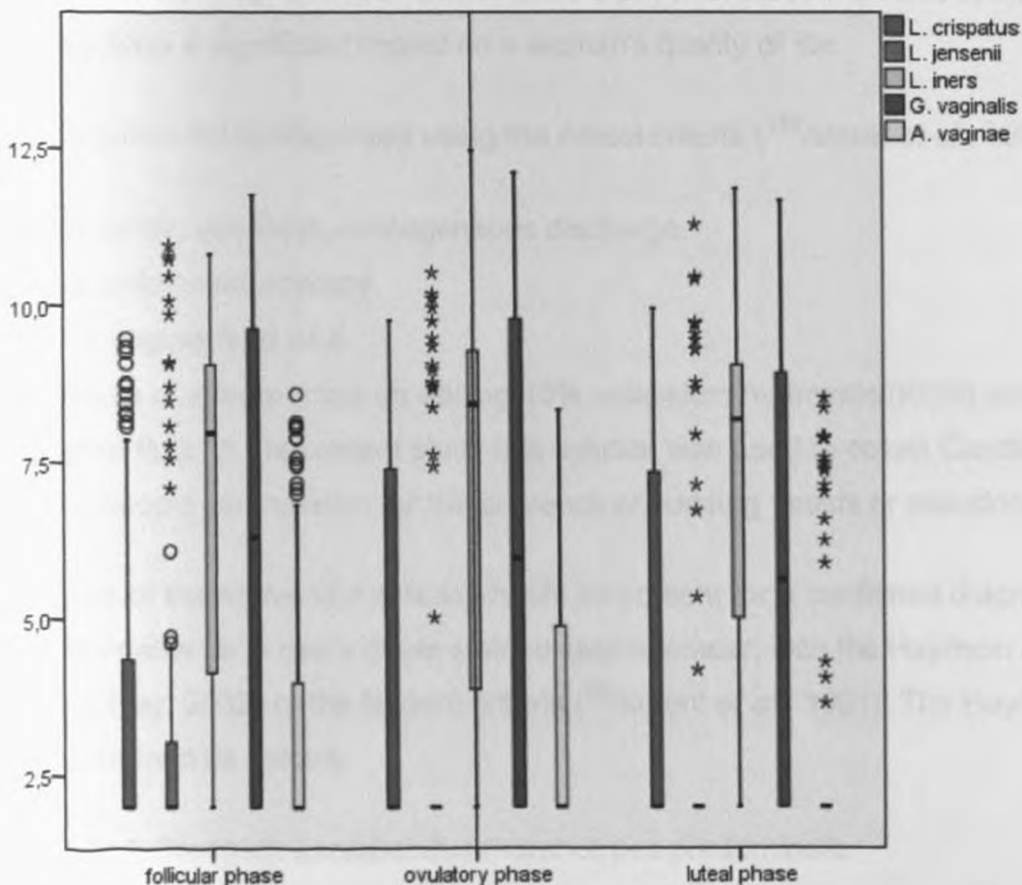


Figure 22- Relative concentrations of *L. crispatus*, *L. jensenii*, *L. iners*, *G. vaginalis* and *A. vaginae* based on phase of menstrual cycle and detected by real time PCR.

CHAPTER FIVE

5.1 DISCUSSION

5.1.1 Introduction

Vaginal microflora is dynamic and it changes in the course of the menstrual cycle. Factors found to have a significant influence in these changes include the use of medication such as antibiotics, contraception, time of the menstrual cycle, the number of sex partners and frequency of intercourse (⁸Marrazo, 2004; ⁵Wilson 2005). Bacterial vaginosis (BV) is twice as common as vaginal candidiasis and it is estimated to be among the most common complaints for which women seek medical help (⁶Nyirjesy, 2008). It has been shown that up to 50% of women suffering from BV remain asymptomatic (⁶Nyirjesy, 2008). In addition to the physical discomfort and symptoms, BV can also have a significant impact on a woman's quality of life.

In clinical practice BV is diagnosed using the Amsel criteria (¹⁵⁹Amsel *et al.*, 1983):

1. Thin, white, yellowish, homogeneous discharge.
2. Clue cells on microscopy.
3. pH of vaginal fluid ≥ 4.5 .
4. Release of a fishy odour on adding 10% potassium hydroxide (KOH) solution to vaginal fluid. In the present study this solution was used to detect *Candida* by microscopic examination for the presence of budding yeasts or pseudohyphae.

At least three of the above four criteria should be present for a confirmed diagnosis of BV. The alternative is to use a Gram stained vaginal smear, with the Hay/Ison criteria (³⁰⁰Ison and Hay, 2002) or the Nugent criteria (⁷³Nugent *et al.*, 1991). The Hay/Ison criteria are defined as follows:

- Grade 1 (Normal): *Lactobacillus* morphotypes predominate.
- Grade 2 (Intermediate): Mixed flora with some Lactobacilli present, but *Gardnerella* or *Mobiluncus* morphotypes also present.

- Grade 3 (BV): Predominantly *Gardnerella* and/or *Mobiluncus* morphotypes. Few or absent Lactobacilli (¹³⁹Hay *et al.*, 1994).

What this technique loses in inter-observer reliability, it makes up in ease and speed of use (¹³⁹Hay *et al.*, 1994; ⁶Nyirjesy, 2008). The standard method for use in research is the Nugent Criteria (⁷³Nugent *et al.*, 1991; ⁶Nyirjesy, 2008), considered to be the gold standard for diagnosis of BV (¹⁴Demba *et al.*, 2005; ⁶Nyirjesy, 2008). In these criteria a score of 0-10 is generated from combining three other scores. It is time consuming and requires trained staff but it has high inter-observer reliability:

- 0–3 is considered negative for BV.
- 4–6 is considered intermediate.
- 7+ is considered indicative of BV.

This is one of the methods used in the current study. Also used in the present study is a more distinctive method of grading the vaginal smears which was developed recently (³¹Verhelst *et al.*, 2005) and allows for a more refined assessment. Untreated BV may cause serious complications, such as increased susceptibility to STIs including HIV and Gonorrhoea (¹¹Martin *et al.*, 1999), *C. trachomatis* and *T. vaginalis* (¹⁰⁹Kaul *et al.*, 2007), and may present other complications for pregnant women (³⁸⁶Ugwumadu, 2002). It has also been associated with an increase of pelvic inflammatory disease (PID) (²⁷⁴Ness *et al.*, 2005). Bacterial vaginosis can be treated with metronidazole and clindamycin (⁶Nyirjesy, 2008). However, there is a high rate of recurrence (²⁶³Bradshaw *et al.*, 2006), which may lead some women to ignore seeking treatment.

The vaginal microflora (VMF) affects the health of the woman, her fetus and newborn as well as her sexual partner. Culture-based identification methods have certain limitations, and this led to the consistent failure to detect some fastidious vaginal bacteria, leading to an incomplete picture of the diversity of the VMF. DNA-based identification methods have facilitated the detection and identification of bacteria without cultivation, although this approach too has inherent advantages and limitations.

Molecular studies of the VMF led to the discovery of several uncultivable bacterial species. An important new insight which stems from this research effort is the discovery that there is a strong association between BV and the presence of a thus far unrecognized species, *Atopobium vaginae* (²⁵⁹Rodriguez-Jovita *et al.*, 1999; ³⁰Verhelst *et al.*, 2004), which is strictly anaerobic and a very fastidious Gram positive coccus. Others include *Dialister*, *Leptotrichia* and *Megasphaera* species which were commonly found in women with bacterial vaginosis. Another research group (²⁵⁶Fredricks *et al.*, 2005) found that several unknown bacteria in the order *Clostridiales* were found to be highly specific indicators of bacterial vaginosis. They suggested that these might be possible candidates for future PCR-based diagnosis of bacterial vaginosis. In addition, the comparison of traditional methods of microscopy and culture with DNA-based techniques for characterization of the vaginal microflora have allowed for the re-definition of the Gram stain based grading of vaginal smears (³⁰Verhelst *et al.*, 2005). This latter group came up with sub-categories within what was previously designated as grade I normal microflora, and identified a distinct cell type related to *L. crispatus* (grade Ia). They described an additional new category (grade I-like) found to be predominated by the presence of *Bifidobacterium* spp.

Both culture and molecular methods have their specific advantages and disadvantages and both are prone to different types of biases, which are not always easy to avoid and which have to be taken into account when analyzing results. Some problems could arise at the time of sample collection, during transport, handling and storage of specimens. Different sites of sampling (cervix, fornix, outer vaginal canal) (³⁸⁷Cosentino *et al.*, 2003), various collecting devices (dacron, flocked or cotton swabs combined with a transport medium such as liquid Amies medium or agar) and different sampling techniques (cervicovaginal lavages, scraping, swabbing) (³⁸⁸Kim *et al.*, 2009) can give different results, which could account for important differences in outcomes regarding the composition of the microflora. According to what has been named 'the great plate count anomaly' (³⁸⁹Relman, 1999), we know that only a very limited part of the microbiome is cultivable and, even this part is further limited by the choice of culture medium and microbial density present in the sample. For example *L. jensenii* forms

larger colonies on Schaedler than on Columbia agar, which results in an easier isolation of *L. jensenii* from the former medium, but on the other hand it is more difficult to isolate other species on Schaedler agar from a sample mainly colonized with *L. jensenii*. In addition, there is the example of *L. iners*, which does not grow on MRS medium routinely used for detection of lactobacilli in vaginal samples and, which therefore has been overlooked in many culture-based studies (⁹⁹De Backer *et al.*, 2007).

Another important consideration is that the biochemical tests for identification of *Lactobacillus* spp. are not always forthright, which often leads to misidentification or lumping them together in larger groups not corresponding to individual species. These problems can be partially tackled by combining different media and coupling this with molecular identification techniques. For molecular techniques, bias could emanate from the use of different DNA extraction methods or from the fact that the DNA of Gram negative bacteria is much easier to isolate than that of Gram positive ones (³⁹⁰Fredricks and Relman, 1999), owing to the thick peptidoglycan layer of the latter. Pretreatment of the sample is necessary and needs to be optimized for the target species. This needs to be compatible with the sample collection device used, for example PCR is not always compatible with a swab transported in non-fluid medium although these swabs give better results for Gram staining. Furthermore, following DNA extraction, the PCR-method can be biased by the choice of primers and targets. ³⁰Verhelst *et al.* (2004) have shown that universal primers are not always as universal as intended. For example the universal 16S rRNA gene-based primer set they applied did not pick up *G. vaginalis* due to three mismatches with the forward primer. This also makes it impossible to quantify due to the large difference in efficiency for the different species. ⁹⁹De Backer *et al.* (2007) also experienced a similar problem with a real-time PCR assay for *Lactobacillus* and *Bifidobacterium* spp. Quantification with genus-specific primers becomes more complicated due to the different PCR-efficiencies for the species belonging to the genus. Mathematical approaches to compensation (³⁹¹Liu *et al.*, 2009) are not useful since they are specific for each sample, which makes the analysis almost impossible. Another potential problem with PCR is primer specificity; for example with the primers for amplification of *L. jensenii*, ⁹⁹De Backer *et al.* (2007) discovered that an

aspecific reaction in some samples occurred due to the simultaneous amplification of an undefined fragment present in the *L. iners* genome. The use of a hydrolysis or hybridization probe, so that only specific amplification is reported for the real-time PCR, could solve this problem. Culture and molecular techniques do not always give similar results due to these biases. Using both techniques (for example culture on different media and tRFLP or real-time PCR) generates large data tables, which are not completely comparable and even often very different due to the different efficiencies of the techniques for different species, making data interpretation difficult. For example *L. jensenii* grows more easily on most media when compared to *L. crispatus*, but when using PCR the number of *L. crispatus* appears to be higher than that of *L. jensenii*. This was encountered in the preset study where culture results showed *L. jensenii* to be predominant in HIV+ve patients while PCR results indicate the same species as almost undetectable in the same group. Newer techniques such as metagenome analysis based on pyrosequencing can generate a broader overview of the vaginal microflora, but the PCR bias remains. To minimize the efficiency bias as much as possible, a combination of primer sets can be applied.

It is clear that molecular analysis of bacterial vaginosis-related organisms has contributed to a more refined description of normal and disturbed VMF. However, it remains to be seen whether these efforts will lead to the elucidation of the elusive etiologic agent of BV. Recent studies have shown that culture remains essential to analysis of VMF. To obtain the most complete picture of the composition of complex microbial communities, studies require the use of a combination of culture-based and culture-independent methods. From the progressive findings of the last five years in this field, it is obvious that a better understanding of vaginal microbial populations will lead to improved strategies on how to maintain healthy VMF, and possibly lead to better ways to explore the role of previously unknown bacteria in reproductive tract diseases. When performed under proper incubation conditions and media, culture tends to be more sensitive than broad-range PCR-based techniques. The development of selective media for fastidious species is essential so that extensive biochemical characterization and investigation of their role in urogenital tract diseases can be investigated. The

current study used a combination of culture and quantitative PCR techniques to investigate the VMF of HIV-infected and HIV-uninfected women in a sub-urban population of Kenya.

5.1.2 Cohort Characteristics

The baseline demographic characteristics of 41 HIV+ve and 33 HIV-ve women are presented in Table 4.1 of the results section. All the participants were sufficiently literate to follow the appointment schedule. For most parameters measured at enrolment, no significant differences existed in the two groups. As expected, the HIV+ve women had lower CD₄ counts and condom use was more common among them compared to the HIV-ve women. Another difference in the groups directly related to the HIV-status was the high antibiotic prophylaxis intake among the HIV+ve group. The Kenya national guidelines for treatment and care for HIV persons allows continuous antibiotic intake for prevention of recurrent bacterial infections. Furthermore, significantly more of the HIV+ve participants were married compared to the HIV-ve ones, and the HIV+ve women had lower levels of schooling.

Co-trimoxazole (trimethoprim-sulfamethoxazole) is the antibiotic approved by the Kenya Ministry of Health as antibiotic prophylaxis and is widely available in public health centres where it is issued free of charge to HIV+ve persons. It has antimalarial activity and is also effective for treatment of and prophylaxis against *Pneumocystis pneumonia* (³⁹²Walker *et al.*, 2010). Current WHO guidelines recommend that co-trimoxazole prophylaxis be given to all symptomatic adults with CD₄ counts lower than 350 cells per μL in resource-limited settings (³⁹³WHO guidelines, 2006). Co-trimoxazole prophylaxis is thought to act partly by reducing risk of mortality due to bacterial infections. Two recent studies have shown there to be significant reduction in mortality and malaria from daily co-trimoxazole prophylaxis in addition to benefits conferred by ART (³⁹²Walker *et al.*, 2010). But in Kenya it is not restricted to those on ART; it is available to all HIV-infected persons irrespective of CD₄ count.

The use of co-trimoxazole prophylaxis resulted in 46% reduction in mortality (³⁹⁴Mermin *et al.*, 2004). Similar studies in Africa found a reduction in morbidity and mortality as well

(³⁹⁵Wiktor *et al.*, 1999; ³⁹⁶Badri *et al.*, 1999; ³⁹⁷Anglaret *et al.*, 1999; ³⁹⁸Badri *et al.*, 2001; ³⁹⁹Zachariah *et al.*, 2003). The benefits seen in the use of co-trimoxazole could be the result of a stabilised immune status. By directly preventing some opportunistic infections, co-trimoxazole might have led to reductions in the frequency of episodes of increased HIV viral load associated with acute illness (⁴⁰⁰Donovan *et al.*, 1996; ⁴⁰¹Bush *et al.*, 1996; ⁴⁰²Mole *et al.*, 1997; ⁴⁰³Hoffman *et al.*, 1999), resulting in improved long-term CD₄-cell response. In Uganda, the cost for an annual supply of daily co-trimoxazole prophylaxis is £6 per person. This low-cost, effective, readily available, and relatively non-toxic intervention has become a basic component of HIV/AIDS care throughout the world (⁴⁰⁴Hamer and Gill, 2008).

5.1.3 Culture-based Characterisation of the Vaginal Microflora

The strength of the present study on the characterization of the VMF of Kenyan women is in the combined use of classical microscopy, cultivation and PCR-based culture-independent methods to study the same samples. Each of these techniques contributes to establishing the composition of a complex microbial population. Both culture and molecular techniques have their advantages and limitations when studying the composition of the complex VMF. The real time PCR results were largely in agreement with the Gram stain score of the VMF. Culture results were also entirely in agreement with Gram stain scores in most of the cases.

Of the 51 species identified by culture and t-DNA PCR, 9 were *Lactobacillus* spp. The t-DNA-PCR method (Welsh and McClelland, 1991; Vaneechoutte *et al.*, 1998; Baele *et al.*, 2002) made it possible to identify the vaginal *Lactobacillus* species rapidly and with high discriminatory power, whereas lactobacilli remain difficult to identify with biochemical means. The poor taxonomy and the identification difficulties have hampered until very recently the study of the distribution of the different species in vaginal samples. As a consequence, thus far only the presence of lactobacilli in general was considered in most studies. However, existing publications (⁵⁹Antonio *et al.*, 1999; ²⁶Vallor *et al.*, 2001; Verhelst *et al.*, ³⁰2004; ³¹2005; ⁴⁰⁵2006; ³²Verstraelen *et al.*, 2004)

clearly indicate the important differences between the different vaginal lactobacilli with regard to their presence in normal and disturbed VMF. *L. crispatus* was found to be associated with normal VMF, in accordance with previous reports (⁵⁹Antonio *et al.*, 1999; ²⁶Vallor *et al.*, 2001), while *L. gasseri* and *L. iners* are present in disturbed VMF as well (⁴⁰⁵Verhelst *et al.*, 2006) and *L. gasseri* bears a strong association with *G. vaginalis* (⁴⁰⁵Verhelst *et al.*, 2006). Recent research has made clear that identification of lactobacilli has to be made at the species level to better understand the etiology of the disturbance of the VMF. Indeed, one of the hypotheses put forward regarding the etiology of BV is the disappearance of the 'right' lactobacilli, possibly by competition with other *Lactobacillus* species (⁴⁰⁶Blackwell, 1999; ⁴⁰⁷Onderdonk *et al.*, 2003).

5.1.4 tDNA PCR and Capillary Electrophoresis

Because the tDNA-PCR technique is rapid, accurate, reproducible (⁴⁰⁸Baele *et al.*, 2000a) and widely applicable, it can be used in routine settings for the identification of bacterial pathogens as well as for large scale identification of screening studies yielding multiple unknown isolates. Once an adequate library has been constructed, no prior knowledge on taxonomy is needed and unknowns can be clustered into groups with similar tDNA-fingerprints.

The applied protocol is applicable to most bacterial species, both Gram-positive and Gram-negative. However, with some species, no or only few amplicons are obtained. Using corynebacteria and mycobacteria, low number peak patterns are obtained. This is most likely a problem of non-universality of the 'universal consensus' primers. Our study group are currently trying to develop primers which enable amplification of strains of these species as well. In addition, as might be expected, some highly related species show indistinguishable patterns.

If the same type of equipment and protocol are used, tDNA-PCR libraries are fully exchangeable between laboratories, as has been shown in an extensive reproducibility study (⁴⁰⁸Baele *et al.*, 2000a). However, due to migration differences between types of capillary electrophoresis equipment, different machines will yield differing tDNA-PCR

patterns for the same bacterial strain, even using the same PCR product. This is mainly attributed to the different fluorescent dyes used. These molecules delay or enhance differently the migration speed of the PCR products during electrophoresis, resulting in different calculation of relative amplicon lengths.

As tDNA-PCR only requires PCR amplification, fast denaturation of fragments, and automated electrophoresis, electropherograms can be obtained within hours of colony picking. Clinical microbiology laboratories need to culture micro-organisms to test for susceptibility on the basis of an antibiogram, making it difficult or even impossible to completely replace culture by genetic approaches. The need to start tDNA-PCR from isolated colonies is thus not a major drawback (³⁴⁸Vaneechoutte *et al.*, 1998). Further, cultured organisms (i.e., complete genomes) are needed to carry out genotyping for the basis of techniques like RAPD, PFGE and AFLP for epidemiological purposes. tDNA-PCR can also be used directly on clinical samples which can be expected to be infected by one single species (e.g., sterile clinical samples, like blood or cerebrospinal fluid). In addition to being useful in routine identifications of human or veterinary pathogens, tDNA-PCR can also be used for identification of large numbers of isolates, e.g., in studies of complex microbiota (⁴⁰⁸Baele *et al.*, 2000a; ³¹Verhelst *et al.*, 2005), or to group single unidentifiable isolates for further taxonomical studies, leading in several cases to the description of new species (⁴⁰⁹Devriese *et al.*, 2005).

The use of tDNA-PCR, in combination with electrophoresis techniques which result in digitized fingerprints, is an approach which can be used to identify numerous bacterial species in a rapid, affordable and reproducible manner. Reliable identification depends completely upon the availability of databases consisting of high quality tDNA-PCR fingerprints obtained from correctly identified strains. Because tDNA-PCR fingerprints obtained on the same type of electrophoresis platform can be exchanged between laboratories, it is possible to construct and continuously expand and improve such online databases through the collaboration between different laboratories. However, the exchange of fingerprints obtained by the use of different electrophoresis platforms will remain problematic or impossible.

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5.2 Changes in the Vaginal Microflora during the Menstrual Cycle

This is the first study to use a combination of Nugent scores and real time PCR to investigate VMF of African HIV+ve and HIV-ve women with and without BV. Vaginal colonization by lactobacilli is believed to confer multiple benefits to women, among them being the inhibition of the development of BV, a condition associated with many undesirable effects such as preterm delivery (²⁶⁶Hillier *et al.*, 1995; ⁴¹⁰Leitich *et al.*, 2003; ²⁷Wilks *et al.*, 2004) and increased vulnerability to other STIs (²⁸⁰Cherpes *et al.*, 2003; ¹⁰⁹Kaul *et al.*, 2007; ⁴¹¹Allsworth *et al.*, 2008), including HIV infection (⁴¹²Cohen *et al.*, 1995; ¹²Sewankambo *et al.*, 1997; ¹⁶⁸Taha *et al.*, 1998b; ¹¹Martin *et al.*, 1999). Thus, molecular characterization of the lactobacilli and other bacteria species that colonize the vaginas of HIV+ve and HIV-ve women has a high potential to help in understanding the dynamism of microflora in health and disease. We found interesting patterns of dynamism of the VMF to occur during the menstrual cycle by using real time PCR to quantify *L. crispatus*, *L. jensenii*, *L. iners*, *G. vaginalis* and *A. vaginae*.

5.2.1 Real Time PCR Analysis based on Gram Stain Category

Application of real time PCR to quantify VMF has previously been used by other groups. Molecular quantification eliminates culture bias and provides for objective interpretation of results and also gives more detailed information of resident species irrespective of density, and can lead to the recognition of novel species (²⁵⁹Rodriguez-Jovita *et al.*, 1999; ³⁰Verhelst *et al.*, 2004). It also allows detection of biologically inactive biofilm communities (³¹⁴Swidsinski *et al.*, 2005) that are inevitably missed out by culture methods. In the vagina of healthy premenopausal women, lactobacilli are the predominant bacteria (³¹⁴Swidsinski *et al.*, 2005; ⁸⁸Zhou *et al.*, 2009). But in this study we found a high load of *L. iners* to be equally represented in normal, intermediate and BV microflora. This confirms the findings of ⁹⁹De Backer *et al.* (2007) who found *L. iners* to be increasingly associated with disturbed VMF unlike previously held that lactobacilli are only associated with normal flora. *L. iners* was also present in normal and intermediate Gram stains which is in agreement with ¹⁵Anukam *et al.* (2006) who found *L. iners* to be the predominant lactobacilli in healthy premenopausal Nigerian women

(64% of 241). Other studies have also reported *L. iners* to be one of the predominant lactobacilli in VMF of BV-free Caucasian women in Sweden (⁹⁶Vasquez *et al.*, 2002) and Canada (42% of 19) without symptoms or signs of urinary tract infection (⁹⁵Burton *et al.*, 2003). Although previous studies missed *L. iners* due to culture bias (⁹⁹De Backer *et al.*, 2007), culture-independent studies have recently shown that *L. iners* is one of the predominant *Lactobacillus* in VMF (⁹⁶Vasquez *et al.*, 2002; ²⁵⁶Fredricks *et al.*, 2005; ⁸⁷Zhou *et al.*, 2007). Here we show that *L. iners* is the predominant *Lactobacillus* in these HIV+ve and HIV-ve Kenyan women, irrespective of the Nugent grading of their VMF.

Also in agreement with ⁹⁹De Backer *et al.* (2007) was the finding that *L. jensenii* was present sporadically in all the Nugent grades. There were high levels of *L. jensenii* in half of the women with BV and very low levels in the women with normal VMF. Our results are also in agreement with ¹⁵Anukam *et al.* (2006) who found that in the Nigerian women they studied, very few with normal Nugent scores had *L. jensenii* detected using PCR-denaturing gradient gel electrophoresis (DGGE). In this study we found *L. crispatus* to be either absent or in very low concentration in women with BV. This is in contrast with ⁹⁹De Backer *et al.* (2007) who found *L. crispatus* to be present in varying concentrations in all the Nugent grades using about 50% pregnant Belgian women. This difference could be explained by the fact that in our study specimen collection was based on the menstrual cycle, and most BV cases coincided with the early phase when the condition is most frequent (¹²⁶Morison *et al.*, 2005). It has also been shown that infrequent specimen collection can lead to underestimation of BV or to omission of the dynamism of VMF (³¹²Brotman *et al.*, 2010).

L. crispatus and *L. jensenii* showed an interesting inverse relationship where *L. crispatus* decreases from the normal microflora to almost being undetectable in BV; whereas *L. jensenii* is almost undetectable in normal microflora but increases progressively to peak at BV microflora. This study provides evidence to contradict the currently held hypothesis that both *L. crispatus* and *L. jensenii* must be present in normal VMF. ⁹⁶Vasquez *et al.* (2002) also found *L. crispatus* to be the single

predominant *Lactobacillus* in the vagina. Our findings correspond with those of ⁵⁵Hawes *et al.* (1996) who found that lack of vaginal H₂O₂-producing lactobacilli or presence of only non-H₂O₂-producing lactobacilli were risk factors for acquisition of BV. It appears that *L. jensenii* alone, though known to be a H₂O₂-producer (⁵⁵Hawes *et al.*, 1996; ⁵⁹Antonio *et al.*, 1999), is not protective since it was very low in normal microflora and high in BV. *L. jensenii* has previously been shown to exhibit both poor colonisation strength and weak colonisation resistance compared to *L. crispatus* (⁸³Verstraelen *et al.*, 2009). It is *L. crispatus* that apparently trips the balance in these women. We propose that in these women only *L. crispatus* is critical in vaginal health, and that possibly, the depletion of this *Lactobacillus* contributes to development of BV. It has previously been suggested that existing differences in microbial communities of Caucasian and African women may well account for discrepancies in their susceptibility to BV and other vagina infections (¹⁰Royce *et al.*, 1999; ⁸⁷Zhou *et al.*, 2007). It is notable that all grades of VMF had at least two species of *Lactobacillus*, which is in conformity with the proposal that the function of lactic acid production in VMF is highly conserved (⁸⁷Zhou *et al.*, 2007; ⁸⁶Ravel *et al.*, 2010).

In this study the concentration of *G. vaginalis* increased progressively from normal to reach a peak in BV microflora. This finding is in agreement with that of ⁹⁹De Backer *et al.* (2007) whom, also using real time PCR, found *G. vaginalis* to be present in all grades of the VMF. ⁹⁵Burton *et al.* (2003) using DGGE and sequence analysis found that the presence of *G. vaginalis* did not necessarily exclude *Lactobacillus* from VMF. This contradicts an earlier hypothesis that *G. vaginalis* only occurred where *Lactobacillus* were depleted (¹¹Martin *et al.*, 1999; ¹⁰⁸Baeten *et al.*, 2009). We found a strong positive correlation of occurrence between *G. vaginalis* and *L. iners* irrespective of the Nugent score of the VMF. We propose that these two species possibly contribute to the comparatively higher pH found in women of African descent (¹⁰Royce *et al.*, 1999; ⁸⁶Ravel *et al.*, 2010), which may play a role in the observed high prevalence of BV in this population. ⁸⁶Ravel *et al.* (2010) found that vaginal bacterial communities not dominated by *L. crispatus* tend to have slightly higher pH values. The vaginal communities in this study population were dominated by *L. iners* irrespective of Nugent

grade, which could imply that these women by nature have higher vaginal pH values. This implies that they tend to have a reduced colonization resistance, a factor that could easily predispose to other less resilient species, leading to development of BV. The differences in the *Lactobacillus* communities present in African women as shown in this study and others (¹⁵Anukam *et al.*, 2006; ⁸⁶Ravel *et al.*, 2010) compared to Caucasian women (⁸⁹Eschenbach *et al.*, 1989; ⁵⁵Hawes *et al.*, 1996), coupled with sexual habits and practices may altogether influence the high susceptibility to BV in women of African descent (¹³Bukusi *et al.*, 2006; ⁹Allsworth and Peipert, 2007; ²⁴⁰Brotman *et al.*, 2008b). It has been reported that *L. crispatus*, even when accompanied by the other *Lactobacillus* species, offers significant stability to VMF in contrast to *L. iners* dominated microflora (⁸³Verstraelen *et al.*, 2009).

We found the concentration of *A. vaginae* to be either very low or absent in normal microflora, detectable in intermediate gram stain and increasing progressively during the BV phase. Other recent studies have documented the presence of *A. vaginae* in VMF. Since its discovery (²⁵⁹Rodriguez-Jovita *et al.*, 1999) and subsequent association with BV (³⁰Verhelst *et al.*, 2004; ²⁵⁶Fredricks *et al.*, 2005), some researchers have proposed that *A. vaginae* can reliably be taken as an indicator to diagnosis for BV (²⁶¹Feris *et al.*, 2004a; ²⁶⁰Burton *et al.*, 2005; ⁹⁹De Backer *et al.*, 2007). These suggestions were based on the observation that *A. vaginae* was present in a high percentage of BV patients. ²⁶⁰Burton *et al.* (2005) detected *A. vaginae* in 50% of Canadian BV patients and ²⁶¹Feris *et al.* (2004a) found that *A. vaginae*-specific PCR assays were negative in all women with normal vaginal Gram stains (35 women in total). They suggested that *A. vaginae* is, rarely if ever, a component of normal VMF. ⁹⁹De Backer *et al.* (2007) found high concentrations of *A. vaginae* in grade III samples. In this study, we found in addition that, *A. vaginae* had an inverse relationship with *L. crispatus*. In BV microflora where *A. vaginae* was highest, *L. crispatus* was absent or not detectable, confirming these earlier reports. It is notable that in our results, the PCR analysis for the normal microflora resembles that for the luteal phase, confirming earlier reports that VMF is most unstable in the early phase of the menstrual cycle (⁷Keane *et al.*, 1997; ²⁵Eschenbach *et al.*, 2000; ¹²⁶Morison *et al.*, 2005). The very low presence of

A. vaginae in normal microflora and the subsequent resurgence in BV microflora may be explained by Walker's hypothesis (⁴¹³Walker, 1992) of microbial community structures referred to as "drivers and passengers" theory. It postulates that for an ecosystem to function, there are "driver" species (in our case *L. crispatus*) that strongly influence the community structure where they occur. Other species in the community constitute "passenger" species that have no major influence. Further, ⁸⁶Ravel *et al.* (2010) postulated that undetectable members of a community (eg. *A. vaginae* in normal Gram stain) may serve as a "seed bank" of species whose numbers multiply when favourable conditions arise, as would happen for example in this case when *L. crispatus* becomes depleted for one reason or another (in the BV microflora).

5.2.2 Real Time PCR Analysis based on HIV status

So far only two studies have compared the VMF of HIV+ve and HIV-ve women (⁴¹⁴Spear *et al.*, 2008; ⁹⁸Spear *et al.*, 2011). This is so far the first study to compare the VMF of African HIV+ve and HIV-ve women, sampled severally during the menstrual cycle, using culture-independent methods. All the HIV-ve women had CD₄ counts above 500, except one with 488. About 56.1% of the HIV+ve women had CD₄ counts of 250≥ while the rest had a count above 500, and none were on ART. Analysis of PCR results based on HIV status showed a high concentration of *L. iners* and *G. vaginalis* in both the HIV+ve and HIV-ve groups. Although condom use was significantly higher in HIV+ve women, the number of women harbouring the protective *L. crispatus* as well as the concentration of *L. crispatus* was remarkably lower compared to HIV-ve women. *A. vaginae* was present in both groups but *L. jensenii* was not detectable in the HIV+ve women. ⁹⁸Spear *et al.* (2011) also found *L. jensenii* to be absent or present at relatively low levels in all but one out of 46 women studied. Since *L. jensenii* is known to have poor colonization resistance (⁸³Verstraelen *et al.*, 2009), it is possible that increased biodiversity in HIV+ve women (⁴¹⁴Spear *et al.*, 2008; ⁴¹⁵Kiama *et al. in press*) leads to the displacement of this species. We did not find that HIV infection influences the incidence of BV, which is in agreement with earlier reports (⁴¹⁵Greenblatt *et al.*, 1999; ⁴¹⁶Watts *et al.*, 2006). The observation that both groups of women harboured *L.*

crispatus and *A. vaginae* may explain why we did not find differences in the prevalence of BV between them, since *A. vaginae* was suggested to be a reliable indicator for BV. It appears that *L. iners* and *G. vaginalis* are a fixed structure of the vaginal communities of these women irrespective of the prevailing circumstances. We suggest that the short-term temporal dynamics of vaginal communities are determined by the species proportions of *L. crispatus* and *L. jensenii*, which in turn determine the relative resistance and resilience to the inevitable challenges of systemic homeodynamism, local disturbances as well as pertinent individual behaviours, habits and practices. Given such an ecosystem, opportunistic and pathogenic species will more likely gain a foothold in communities that exhibit low stability (⁴¹⁸Hobbs and Huenneke, 1992; ⁸³Verstraelen *et al.*, 2009). This is a likely situation where *L. iners* predominates, since it is known to offer less colonization resistance due to low H₂O₂ production (⁸⁹Eschenbach *et al.*, 1989; ³²²Hillier *et al.*, 1993; ⁸³Verstraelen *et al.*, 2009), coupled with the observation that such communities tend to have slightly higher pH values (⁸⁶Ravel *et al.*, 2010) and are less stable than those where *L. crispatus* predominates (⁸³Verstraelen *et al.*, 2009).

5.3 Real Time PCR Analysis based on Phase of Menstrual Cycle

Analysis based on phase of menstrual cycle showed a high concentration of *L. iners* and *G. vaginalis* throughout the menstrual cycle. In all the three angles of analysis done here these two species appear present in high concentrations. We propose that they are a fixed structure of the vaginal communities of these women irrespective of the prevailing circumstances. The concentration of *L. crispatus* increased while concentration of *A. vaginae* decreased throughout the menstrual cycle. This trend is exactly a reversal of what we observed in PCR analysis based on Gram stain scores. It has previously been reported that VMF tend to be less stable during the early phase of the menstrual cycle (⁷Keane *et al.*, 1997; ²⁵Eschenbach *et al.*, 2000; ¹²⁶Morison *et al.*, 2005). This instability appears to be coupled to the decrease and subsequent displacement of *L. jensenii* and *A. vaginae* from follicular through to the ovulation phase. *L. jensenii* was not detectable after the initial phase of the menstrual cycle where it was

comparatively low. Dynamic systemic changes in hormonal levels occurring at this time are usually accompanied by dramatic changes in the vaginal epithelium that influence the type and volume of secretions, the pH as well as the adherence capabilities of resident communities in the face of menstrual flooding (¹³¹Owen Jr., 1975; ²⁴⁰Brotman *et al.*, 2008b; ⁶⁰Farage *et al.*, 2009). During the follicular phase all the three lactobacilli studied were present together with *G.vaginalis* and *A. vaginae*. This state of VMF is mirrored by the intermediate Gram stain category. It appears to be a transient stage that the microflora goes through before the full effects of the systemic and local changes take their toll. Immediately after, in the ovulatory phase, instability is seen in the displacement of *L. jensenii* and the accompanying steady concentration of *A. vaginae*. The luteal phase is driven by increased secretion of two groups of steroids, namely progesterones and estrogens (¹³¹Owen Jr., 1975; ⁶⁰Farage *et al.*, 2009). The PCR picture of the VMF during this phase resembles closely the one obtained for the normal microflora where both *L. jensenii* and *A. vaginae* were either absent or undetectable. We provide evidence here that the instability witnessed in the early stage of the menstrual cycle is due to decreasing levels of *L. jensenii* driven by yet undetermined factors, as well as increasing levels of *A. vaginae*.

⁴²⁰Srinivasan *et al.* (2010) observed that during menses, the population of *G. vaginalis* increased alongside that of *L. iners*, while quantities of *L. crispatus* and *L. jensenii* decreased simultaneously. Similar observations were made in an earlier study (⁴²¹Schwebke *et al.*, 1997). The former authors also observed that *G. vaginalis* was present in women with and without BV. *G. vaginalis* contains vaginolysin that can perforate erythrocytes to release iron and also activate immune markers of the vaginal epithelium (⁴¹⁹Gelber *et al.*, 2008), causing the inflammation observed in BV. Vaginolysin belongs to cholesterol-dependent cytolysins produced by organisms that colonize and cause disease at mucosal surfaces (⁴²²Tweten, 2005). The implication is that in these women, inflammation of the vaginal epithelium constantly poses the danger of development of BV. Toxin production has been shown to be essential for maintenance of colonization and pathogenesis of invasive disease (⁴²²Tweten, 2005). Systemic effects of hormones may then only contribute to ongoing inflammation at the vaginal epithelium.

5.4 Prelude on Vaginal Microflora Research

In the late 19th century German gynaecologist Albert Döderlein investigated the occurrence of severe, febrile condition in women following delivery (termed “puerperal fever”) and he attributed this infectious condition to the loss of the indigenous lactobacilli from the lower female genital tract (⁷⁸Döderlein, 1892). During the following century gynaecologists and microbiologists had growing interest in the anaerobic overgrowth of VMF, known since the 1980s as bacterial vaginosis. There have however been intrinsic limitations posed by the traditional culture-based species identification and quantification in documenting the complexity VMF (¹³⁷Kalra *et al.*, 2007). This has in turn also hampered the study of the dynamics of the vaginal bacterial community that is likely to be a key aspect to the understanding of the pathogenesis of BV. Further, with the introduction of molecular techniques which were first applied to environmental samples in the 1990s, it became increasingly apparent that many bacteria were overlooked in the culture-based studies. Surveys of many terrestrial and aquatic ecosystems for instance indicated that >99% of microorganisms resist cultivation in the laboratory (³⁸⁹Relman, 1999). As a consequence microbiological research has for a long time been limited to only species that are cultivable. The advent of molecular techniques has tremendously changed the picture of microbial diversity in existing communities. Although molecular studies of the VMF are still in their infancy stage in relation to studies of environmental microbial ecology and of the gastrointestinal tract (¹³⁷Kalra *et al.*, 2007), several interesting discoveries have been made in recent years. In the studies quoted in this thesis, it was also apparent that the molecular approach proved a useful adjunct to conventional culture techniques, however cultivation techniques remain indispensable to the study of VMF.

As the use of molecular methods continues to increase, it is important to acknowledge that they have inherent biases associated with their use. Pre-analytical bias occurs pertinent to sample collection, handling and storage, and progress in standardisation of these procedures has been slow to come. As we used these handy molecular

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As the use of molecular methods continues to increase, it is important to acknowledge that they have inherent biases associated with their use. Pre-analytical bias occurs pertinent to sample collection, handling and storage, and progress in standardisation of these procedures has been slow to come. As we used these handy molecular

techniques, we were aware of several sources of analytical biases with 16S rDNA-based characterisation.

- (1) Firstly, DNA extraction from clinical samples may bias 16S rDNA amplification due to differential susceptibility of Gram-negative and Gram-positive bacteria to the cell lysis methods. The Gram-positive cell wall is significantly more resistant to cell lysis. This bias can be largely contravened by increasing the efficiency of bacterial cell wall lysis with specific enzymes such as mutanolysin applied in this study.
- (2) Secondly, broad-range or so-called universal 16S rDNA PCR amplification is also susceptible to amplification of bacterial DNA present at low concentrations as contaminating bacterial DNA in reagents, laboratory material, etc. possibly biasing the characterisation of the clinical sample. This contamination phenomenon can be monitored by including blank or 'no template' specimens subjected to PCR in each run to see if the contamination has a significant contribution or not.
- (3) A major limitation with broad-range or universal 16S rDNA primers is also the observation that these primers are generally not as universal as they are intended to be. Several bacteria display polymorphisms at the conserved regions of their 16S rDNA gene. This translates as a mismatch between the primer and the 16S rDNA template, eventually leading to less efficient priming and hence sub-optimal amplification. As a result, the identification and quantification of these bacteria will be negatively biased through deficient sensitivity for selected species. If the position of the mismatching base is known, the primers can be modified with multiple bases or with inosine residues at the complementary position in the primer. However, the increasing use of specific primers is likely to overcome this source of bias.
- (4) Another limitation to the 16S rDNA based approach results from the number of rDNA copies within the genome of a given bacterium. The number of copies ranges from 1 to 13 and those with a higher number of rDNA copies will be amplified more efficiently and to a larger extent, implying that these species will

be overrepresented relative to the species with fewer rDNA copies. So far there is no satisfactory method to overcome this limitation.

- (5) The content of the Guanine/Cytosine (GC) versus Adenine/Thymine (AT) in bacteria rDNA differ significantly. The stronger binding of GC base pairs compared to the AT base pairs results in reduced amplification efficiency of those species with a higher GC content. The addition of reagents such as tetramethylammonium chloride (TMAC) and dimethylsulfoxide (DMSO), which are known to facilitate DNA strand separation, has been applied to minimize this bias.
- (6) Another potential source of bias involves cross-reaction of a primer-induced sequence with two closely related templates resulting in chimera sequences that represent non-existent bacteria. The sequences generated through amplification may also miss parts of the template sequence due to stable secondary structures.
- (7) In a highly diverse bacterial ecosystem as is clearly the case with VMF, the genomic fingerprint will only represent the most abundant species, considering these species occur at several orders of magnitude above others. Newer 16S rDNA-based approaches, such as pyrosequencing display a much higher resolution and sensitivity and are now being introduced for the characterisation of the VMF.

While bearing these limitations in mind, the very recent body of molecular research of the VMF obtained over the past five years may be considered a first step in the unravelling of the complexity of the vaginal microbial ecosystem. Firstly, through 16S rDNA-based analysis it has been established that normal or grade I microflora are dominated by one or more of four *Lactobacillus* species *L. crispatus*, *L. jensenii*, *L. gasseri*, and *L. iners* (⁹⁹De Backer *et al.*, 2007), though we now also have evidence that *L. vaginalis* may have been overlooked. In a number of other studies the VMF has been analysed through various culture-independent techniques in very different populations around the world. This in turn has resulted in wide-ranging point estimates of the prevalences of these distinct lactobacilli. It remains to be determined whether such

differences reflect genuine interpersonal or interpopulation differences. Most studies on 16S rDNA-based analysis did not collect data on behaviour-related study population characteristics, and hence the failure to correlate patient determinants with VMF characteristics. Again, now that we have a more or less clear picture of what constitutes the normal VMF, the doors are open in search for such determinants.

One study indicated that the presence of *L. crispatus*, and to a lesser extent *L. jensenii*, is a prerequisite to a stable microflora, whereas *L. gasseri* and *L. iners* were more conducive to the occurrence of abnormal microflora and BV in pregnant women (Verstraelen *et al.*, 2009). The present study on non-pregnant premenopausal women confirms this, and it may then be inferred that on average, about half of all women carry a rather stable normal microflora, whereas the remainder are more prone to convert to an abnormal VMF. This observation leads to the question of what determines a woman's microflora. Clearly part of the answer to this question lies in the constitution of the *Lactobacillus* species present in the community. More research is needed to investigate genetic, ecologic as well as host immune factors that probably enhance the presence of certain *Lactobacillus* species in preference to others.

Our findings on the vaginal microbiology of the HIV+ve and HIV-ve Kenyan women indicate that *L. iners* is present in high concentration throughout the menstrual cycle. This is also the case in normal and in BV microflora. These findings are relevant to our understanding of the pathogenesis of BV and to its high prevalence in women of black race. Through quantitative real-time PCR we found that growth of *L. iners* is apparently enhanced in BV. Hence, whereas BV is traditionally described as a state of depleted vaginal lactobacilli, we found *L. iners* to persist in the altered vaginal microenvironment with BV as well. This confirms the findings of De Backer *et al.* (2007). As *L. iners* is so abundant during BV, it may be possible that it is consequently the most likely *Lactobacillus* to replenish the vagina following the resolution of BV, a postulate that has recently been corroborated by some evidence though using a small sample size (Fredricks *et al.*, 2005; Ferris *et al.*, 2007). This in turn may render these patients more vulnerable to a new episode of BV, considering the less persistent colonisation

resistance offered by *L. iners*. Preliminary findings support the prevailing hypothesis that recuperation of a stable *Lactobacillus* microflora is crucial to the long-term outcome of BV following treatment, and that the presence of abundant *L. iners* in BV compromises this evolution. Like the above authors we found that occurrence of *A. vaginae* is a typical characteristic of disturbed VMF alongside *G. vaginalis*, possibly indicative of a synergistic relationship between these two species. In addition, a majority of *A. vaginae* strains was found to elicit pronounced resistance to metronidazole, which in turn may concur with the observations that recurrence and therapy failure with BV are strikingly higher among women harbouring *A. vaginae* than those who carry only *G. vaginalis* (De Backer *et al.*, 2007).

Another recent finding that helps to explain the recurrent nature of BV is the discovery that a dense, adherent biofilm of *G. vaginalis* and *A. vaginae* covers the vaginal epithelium in this condition (Swidsinski *et al.*, 2005). In a longitudinal, uncontrolled study of the BV biofilm among a limited number of patients treated with oral metronidazole, members of our study team were able to document that although all patients apparently successfully converted to normal VMF following standard metronidazole treatment, the *G. vaginalis/A. vaginae* BV biofilm was actually only temporarily suppressed by metronidazole and rapidly regained its activity after treatment cessation. As a sophisticated immune and drug escape mechanism, the bacterial biofilm in BV therefore most likely emerges as a primary microbiological correlate of the resistant and recurrent nature of BV.

The effective eradication of BV and hence of its biofilm may in itself not guarantee recolonisation of the vagina with a stable *Lactobacillus* microflora, and recurrence of BV is to some extent also postulated to arise from defective re-colonisation with a “normal” VMF. From this perspective, there has arisen the view by many researchers for the development and use of vaginal probiotics to replenish the vagina with a stable microflora following the application of antibiotics. Few studies have actually looked at the possibility to use probiotics without any pre-treatment, but a number of phase II trials

are now underway, in which the combined effect of antibiotic and probiotic administration on BV cure is under investigation.

New studies are underway and treatment of BV with probiotics is currently regarded as the most promising therapeutic approach to this problem. Several recent studies have indicated for instance that following the largely disenchanting microbicide story, restoration of the VMF is a most promising avenue to tackle the HIV burden in sub-Saharan Africa. Further, the spectrum of VMF alterations is not confined to BV, although it is no doubt the most common manifestation of an abnormal microflora.

⁴²³Donders *et al.* (1998) proposed a revision of the categorisation scheme for the VMF not based on the traditional Nugent scoring scheme, while (³¹Verhelst *et al.*, 2005) introduced a modified classification scheme. These refined classifications may enhance the identification and treatment of patients at risk of preterm birth (⁴²³Donders *et al.*, 1998; ³¹Verhelst *et al.*, 2005). There is need to cross-validate the respective approaches, which might eventually lead to an integration of existing classification schemes.

In conclusion the molecular analysis of the VMF has proven a valuable adjunct to conventional techniques such as Gram stain and culture. Studies conducted through these means over the past five years have provided us with new insights of the VMF, including a better understanding of the role of the vaginal lactobacilli and mechanisms involved in the treatment failure and recurrence of BV. The VMF studies may further translate to improved diagnostics and therapeutics. However, this may be just the beginning of a new research era and more effort will be required to achieve a thorough understanding of the dynamics of the vaginal microbial community.

5.5 Future Perspectives

Molecular techniques have led to the discovery of many new species, which have to be evaluated one by one in characterization and treatment studies. Further studies of the

VMF can not only be done with molecular techniques; it is important to invest in the search for better growth media. For several of the newly discovered species the evidence of their presence is largely molecular, without culture, which is needed to determine their biochemical and antibiotic resistance characteristics. Some of the newly discovered species cannot even be classified or given a name due to the short DNA sequence which is available. The next challenge is to culture these bacteria for further characterization.

For *A. vaginae* it is known that this bacterium might play an important role in the etiology of BV, but exact mechanisms are yet unknown. ²⁶³Bradshaw *et al.* (2006) observed a significant reduction after metronidazole treatment but from one month after treatment, a high rate of recurrence was seen in women with *A. vaginae*. The presence and role of this species in vaginal biofilms and its possible role in the recurrence of BV need to be further investigated. *In vitro* models of the vaginal epithelium and the biofilm might bring new insights to the etiology of BV, although the results need to be interpreted with caution because not all factors influencing the VMF are known or transposable to an *in vitro* model. Again, efficiency in culture of the bacteria will remain crucial.

With the discovery of the new bacteria, old and new treatment options regain the attention of scientists, especially in the perspective of the frequent recurrence of BV. Recently, probiotics, disinfectants, new antimicrobial agents and combinations of treatment regimens have been reconsidered. Recent work on the characterization of the VMF indicates that certain lactobacilli such as *L. iners* and *L. gasseri* may not be ideal for use as vaginal probiotics due to their presence in comparatively less stable VMF. On the other hand, *L. jensenii* and *L. crispatus* are known to be powerful producers of hydrogen peroxide and predominant members of more stable microflora, and may therefore be better suited as probiotics. The *in vitro* characteristics of the bacteria such as antibiotic susceptibility, hydrogen peroxide production and viability (shelf life) of the strain in the formulation are crucial for success. Other important factors to be considered include the capacity for strong adherence to the vaginal epithelium and to proliferate in the vaginal microenvironment. Follow-up studies after administration of

probiotics or antimicrobial agents can be carried out using techniques such as real-time PCR. Recent studies have demonstrated that availability of detailed characterization of the VMF may lead to a better identification of a high risk population for preterm birth. Especially useful is the cheap and easy to perform Gram stain or wet mount, following refinement of their interpretation by combining observations with results obtained by means of molecular techniques.

So far there is yet no molecular method available for a good diagnosis of BV, although *A. vaginae* has been identified as an important marker and, when present together with equally high concentrations of *G. vaginalis*, the sensitivity and specificity rises (²⁶³Bradshaw *et al.*, 2006; ²⁵⁸Menard *et al.*, 2008). The use of more elaborate techniques such as micro-arrays and pyrosequencing still holds some potential in the search for new makers that could give a more accurate diagnosis of BV and of populations at risk for preterm birth.

The question of whether BV is transmitted sexually remains controversial. The concept of BV as a sexually enhanced disease as gained some ground (²³⁷Verstraelen, 2008). This implies that sexual activity increases the disturbance thus creating the right conditions that allow anaerobes to displace the normal vaginal lactobacilli. New or yet-to-be discovered microorganisms might play a role in the etiology and recurrence of BV. The long-unanswered question of whether the decrease in lactobacilli associated with BV precedes the evident overgrowth of anaerobic bacteria or vice versa may find an answer in molecular techniques. These methods will need to be quantitative and very sensitive in order to cover a large range of species. The factors that determine a healthy VMF, be they genetic, physiologic or immune related, remain elusive. There is clear indication even from the current study, that *A. vaginae* may play a significant role in the etiology of BV, but exact mechanisms are yet to be deciphered. The recent detection of so many new vaginal species by means of molecular methods may require earlier studies to be reconsidered, while new and upcoming ones need to take into consideration these new species. The real-time PCR assays developed by ⁹⁹De Backer *et al.* (2007) have in fact been applied with good outcomes to compare vaginal and

rectal microflora (⁴²⁴El Aila *et al.*, 2010). Future research aimed at developing effective therapeutic strategies will be guided by the existing body of knowledge. An *in vitro* *G. vaginalis* biofilm model has recently been developed and could be particularly useful in this respect, albeit difficult to validate, due to differences between *in vitro* and *in vivo* microbial community dynamics.

5.6 Conclusions

The rate of BV was found to be equally high in both HIV+ve and HIV-ve women. Normal, intermediate and BV episodes were evenly spread out in the three sampling points. Minor differences in the species composition of the VMF of HIV+ve and HIV-ve women were found. *L. crispatus*, *L. iners*, *L. jensenii* and *L. vaginalis* were the predominant lactobacilli in culture. The load of the protective *L. crispatus* is lower in HIV+ve women despite significant higher condom use.

Here we show that *L. iners* is the predominant *Lactobacillus* in these HIV+ve and HIV-ve Kenyan women, irrespective of the Nugent grading of their VMF. This study provides evidence to contradict the currently held hypothesis that both *L. crispatus* and *L. jensenii* must be present in normal VMF. We propose that only *L. crispatus* is critical to the health of vagina, and that possibly, the depletion of this *Lactobacillus* contributes to development of BV. We suggest that *L. iners* and *G. vaginalis* are a fixed structure of the vaginal communities of these women irrespective of the prevailing circumstances.

Our results showed that *A. vaginae* had an inverse relationship with *L. crispatus*. In BV microflora where *A. vaginae* was highest, *L. crispatus* was absent or undetectable. In reverse the concentration of *L. crispatus* increased while concentration of *A. vaginae* decreased throughout the menstrual cycle. We confirm that the instability observed in the VMF during the early phase of the menstrual cycle is coupled to the decrease and subsequent displacement of *L. jensenii* and *A. vaginae* from follicular through to the ovulation phase.

5.7 Recommendations

- The load of the protective *L. crispatus* was lower in HIV+ve women despite significant higher condom use. Since only this *Lactobacillus* appears to determine the health of the vaginal microflora, further studies are required to determine the immune factors that influence its sustenance.
- We propose that only *L. crispatus* is critical to the health of vagina, and that possibly, the depletion of this *Lactobacillus* contributes to development of BV. The development of probiotics targetting this population needs to consider that only repopulation with *L. crispatus* may be required to restore vaginal health.
- The concentrations of *L. iners* and *G. vaginalis* remained consistently high in cases with and without BV, a clear indication that they are a fixed structure of the vaginal communities of these women and need to be considered as such.
- The long-unanswered question of whether the decrease in lactobacilli associated with BV precedes the overgrowth of anaerobic bacteria or vice versa remains begging. With molecular techniques at our disposal, further studies are required to decipher the factors that determine a healthy VMF, be they genetic, physiologic or immune related.
- Although the etiology of BV remains undetermined, there is clear indication from the current and previous studies that *A. vaginae* may play a significant role. The exact mechanisms are yet to be deciphered.
- The presence and role of *A. vaginae* in vaginal biofilms and its implication in the recurrence of BV need to be further investigated. *In vitro* models of the vaginal epithelium and the biofilm might bring new insights to the etiology of BV, although the results need cautious interpretation since not all factors influencing the VMF are known or transposable to an *in vitro* model.
- We found the concentration of *A. vaginae* to be inversely proportional to that of *L. crispatus*. In BV microflora where *A. vaginae* was highest, *L. crispatus* was absent or undetectable. In reverse the concentration of *L. crispatus* increased while concentration of *A. vaginae* decreased throughout the menstrual cycle.

Studies of this nature with a longer follow up period will help draw firm conclusions on the interaction of these species.

- We confirm that the instability observed in the VMF during the early phase of the menstrual cycle is coupled to the decrease and subsequent displacement of *L. jensenii* and *A. vaginae* from follicular through to the ovulation phase. Since some women do recover from this instability while others proceed to BV, there is need to design studies that could determine interventions that predispose to remission.
- Due to cost constrains, not all the *Lactobacillus* species identified could be quantified by real time PCR, yet these patterns would be interesting to compare with known trends in the course of the menstrual cycle.
- Although basic information about sexual behaviour was available for the present cohort, future studies need to combine molecular characterization of the VMF with detailed sexual and social and nutritional characteristics of participants in the quest to find the determinants of the VMF.
- The 2007/ 2008 post-election aftermath contributed to some loss to follow-up as some participants were affected and relocated from Tigoni area.
- There is need to include in health dissemination policy the importance of healthy vaginal microflora and the need to protect the same.
- This study has availed for the first time data that spans the full spectrum of VMF of African women which will be very useful in the design of interventions such as probiotics, microbicides and topical antimicrobials.

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APPENDIX 1

CLIENT INFORMATION AND CONSENT

Q. Why is the study being done?

A. Researchers from the University of Nairobi will be conducting a study at Tigoni sub-District hospital. This study is being done in order to help us understand the factors that cause the HIV virus to find it easy to be transmitted and infect some women and not others. We hope we can be able to help all people protect themselves effectively against this infection, for which there is currently no known (medical) cure.

The main purpose of this study is to determine and characterize the vaginal microflora in women of reproductive age.

Q. Who will participate in the study?

A. All women in this (Tigoni) area aged 18 to 45 years who are not pregnant and are able and willing to make the decision, are invited to participate.

Q. How many women will participate in this study?

A. The study needs a total of 100 women in Tigoni area who are HIV negative and HIV positive, which means that no one can know your status just because you participate in this study. There may be some women who will not meet our inclusion criteria and will be excluded even though they are willing to participate. The inclusion criteria are:

1. Normal Pap smear result within the last year
2. Age between 18-45 years
3. Regular menstruation at least 2 cycles or more
(Length of cycle to be 25-35 days)
4. Not pregnant (do a pregnancy test)
5. Not using hormonal contraception
6. Never been exposed to HAART
7. Uterus and cervix intact
8. No systemic disease the last 3 months
9. No delivery or abortion in the last 3 months:
10. Client in good health
11. CD4 Count ≥ 250 cells/ μ L in last 3 months
12. Willing to participate
13. Available to come to the clinic on a weekly basis for two months

14. Mentally competent to give informed consent

Q. What is involved in participating in the study?

A. Women who are willing to participate in the study will be required to come for a medical examination once per week for 8 weeks at the study clinic and to answer some questions. Thereafter some blood specimens and some vaginal swabs will be taken, followed by a cervicovaginal lavage (wash).

During the following visit results of the laboratory tests will be discussed and, if there is an abnormal one the client will be given advice on what to do.

We would like to maintain contact with every participant for the next approximately 10 weeks from the time of enrolment to ensure that she remains well and that should she have any problem, we can detect it in good time and do something about it. Today we will only take blood samples, endocervical and vaginal swabs.

For those who qualify to participate we shall reimburse bus-fare expenses at each visit. Some specimens will be taken for analysis to collaborators outside Nairobi and also outside Kenya and will not bear the names of the patient. Other specimens will be archived for further studies.

When the results of the research are published, the subjects' identity will remain confidential.

Q. What are the risks of the study?

A. The study team will do everything possible to minimize the risks to the study clients who participate in this study. The needle for drawing blood is sterile and will be used only once and then be discarded. Some discomfort may be experienced at the site of injection to draw blood.

In the gynaecological examination everything used is sterile and for single use only. The liquid used for vaginal wash is sterile and can as well be used by the doctor during surgery. Some spotting may occur afterwards, but clients will be given hygiene towels to take care of this.

The blood test results may cause some anxiety but everything will be explained before any test is done and what it means to the woman and her health. Clients will also be counselled by someone qualified to give advice on health matters.

Q. What are the benefits of participating in the study?

A. By participating in the study the woman gets a free medical examination and screening for sexually transmitted infections, blood pressure and anaemia.

In addition participants will also have a test to check whether or not they are infected with HIV if they have not done the test before. This information is very important because if one is not infected they will know how to reduce the risk of infection. And if one is HIV positive, they will be informed on what they should do to remain healthy and also how to protect their partner from being infected if he is negative. Clients will also have access to information on new developments in the treatment of HIV infection and other sexually transmitted diseases. Those who qualify for antiretroviral treatment will be referred to other members of this group (University of Nairobi Institute of Tropical and Infectious Diseases, UNITID) who will be giving treatment for HIV infection. They may also benefit indirectly from the results of this study because the information obtained will help scientists develop better ways of preventing infection with HIV and perhaps also how to help those already infected.

Q. What are my rights as a participant of the study?

A. Very strict confidentiality will be observed all throughout this study. All information obtained about our clients will be locked up in a cupboard to which only the researchers will have access. The specimens taken for testing will have a number and not the client's name on them.

The information published about this work will also not bear the client's name, and personal identity will be protected at all times. Participation in the study is voluntary and no one will be penalized for refusal to participate.

Q. HIV Pre-test Counselling in the study

A. Everyone has the right to know their immune status in order to protect themselves and those they care about from getting infected with HIV. The information received about HIV status and other sexually transmitted diseases, and about reproductive health in general, should help our clients as individuals as well as their partners. The information received will definitely help our clients overcome the anxiety usually associated with not knowing one's immune status.

Early detection of HIV infection is an advantage because it allows the person to have access to sources of support and a variety of treatments available before it is too late. Women also obtain information on how to protect their unborn baby should they decide to have one, as well as information on prevention and treatment of opportunistic infections associated with HIV and AIDS.

Any information about the HIV status of our clients will be kept strictly confidential and will not be released to any other person, including the spouse or other study participants, unless the client has authorised it.

Q. HIV Post-test Counselling in the study

A. To some people a positive test result may generate feelings of shame and even guilt. This makes it difficult for them to be able to reveal the test result to their partner or family. Our counsellor will help the clients to address their immediate concerns and be able to make decisions about disclosure and identify sources of support in their families and communities. Referring patients to existing HIV support groups, and in particular those of people living with HIV/ AIDS, has proved to be especially helpful in critical times.

Information given to HIV positive clients will include the stage of the infection, other infections and illnesses associated with AIDS and available options for treatment. The counsellor will educate the client about antiretroviral (ARV) drugs. The client will also be informed about good nutrition and the importance of obtaining prompt treatment for any opportunistic infections and especially tuberculosis.

Some women who are HIV positive may still want to get children. Accurate information will be given on HIV/AIDS and pregnancy, how to protect their own health and how to avoid transmitting the virus to their baby, as well as information on family planning.

Q. Whom do I contact if I have any concerns or questions about my participation in the study?

A. This study has been approved by the Ethics and Research Committee of the University of Nairobi and Kenyatta National Hospital. Clients may contact the Chairman of the committee, **Tel 272-3600**.

The study team is also available to discuss any concerns arising during the visits to the clinic. Clients may also contact the staff from Reproductive Health Project, University of Nairobi:

Eunice Tel: 0721-727 300 or 0733-875 602

Anne Tel: 0722-398 657

Teresa Tel: 0722-394 190

OR Reproductive Health Project Leader and Director, UNITID, University of Nairobi.

Prof. B. Estambale Tel: 272-6765.

APPENDIX 2

HABARI JUU YA UTAFITI KWA WASHIRIKI

Swali: Utafiti huu unahusu nini?

Jibu: Watafiti wa Chuo kikuu cha Nairobi ndio watakaofanya utafiti huu. Utafiti huu utatusaidia kuelewa vyema zaidi ni kwa nini virusi vya ukimwi vinawadhuru watu mbalimbali. Tunatarajia tutaweza kuwasaidia watu wengi kujikinga na ugonjwa huu hatari kwani hakuna uponyaji wa kisayansi uliogunduliwa mpaka wakati huu.

Makusudi ya utafiti huu ni kuchunguza hali ya chembembe za kuzuia magonjwa katika njia ya uzazi.

Swali: Kina nani watakao idhinishwa kuhusishwa na utafiti huu?

Jibu: Kina mama wote katika eneo hili la Tigoni ambao wana umri kati ya miaka 18 na miaka 45 na ambao kwa wakati huu sio wajawazito na pia ambao wangependelea kuhusishwa. Wote wamealikwa.

Swali. Ni wanawake wangapi watakoa husika na mpango huu?

Jibu: Utafiti huu unahitaji wanawake 100 kutoka eneo la Tigoni, kati yao walioadhiriwa na wasio adhiriwa na virusi vya ukimwi. Hivyo basi hakuna atakayeweza kutambua hali yako kwani wanawake wote watahusishwa. Hata hivyo kuna baadhi ya wamawake ambao hawatahitimu kiwango cha kuhusishwa, ingawa wangependa kuhusika. Washiriki watahitajika kutimiza viwango vifuatavyo:

1. Kipimo cha chembechembe zilizomo katika mlango wa nyumba ya uzazi kiwe sawa katika kipindi cha mwaka mmoja uliopita.
2. Umri kati ya miaka 18 na 40.
3. Damu ya mwezi iwe inakuja inapotarajiwa (iwe kati ya siku 25 na 35).
4. Asiwe mja mzito (Atafanyiwa kipimo cha maabara).
5. Asiwe akitumia dawa za kupanga uzazi.
6. Hawajatumia dawa za ukimwi kamwe.
7. Nyumba ya uzazi iwe sawa.
8. Asiwe amegonjeka kwa muda wa miezi 3 iliyopita.
9. Asiwe amejifungua kwa muda wa miezi 3 iliyopita.
10. Awe mwenye afya bora.
11. Awe anapendelea kushiriki.
12. Awe anaweza kutembelea kliniki mara moja kwa juma kwa muda wa majuma manane.

Swali: Wahusika watahitajika kufanya nini?

Jibu: Wanawake watakaoshiriki katika utafiti watahitajika kuzuru kiliniki kufanyiwa uchunguzi mara moja kwa wiki kwa muda wa majuma manane. Kila wanapotutembelea watapimwa damu na kufanyiwa uchunguzi mwengine. Pia washiriki watajadiliana matokeo ya uchunguzi na kupewa mawaidha jinsi ya kutibiwa. Tutahitaji kuwasiliana na wahusika kwa muda wa majuma kumi kwa jumla. Kukiwa na shida yoyote washiriki watatibiwa ipasavyo. Kila washiriki wanapokuja watarudishiwa gharama yao ya nauli.

Viungo fulani vya wagonjwa vya utafiti vitasafirishwa sehemu zingine nchini na hata nchi za ng'ambo, na pia kuhifadhiwa na kutumika kwa utafiti baadaye.

Viungo hivyo vya utafiti havitakuwa na majina ya washiriki. Matokeo ya utafiti yatakapo chapishwa majina ya washiriki hayatafichuliwa.

Swali: Kuna hatari gani kuhusika na utafiti huu?

Jibu: Hakuna hatari yoyote ya kufadhaisha washiriki. Vyombo vyote vinavyotumika ni safi kadili yake. Kila chombo kinatumika mara moja na kutupwa. Washirika watatunzwa vilivyo kulingana na mahitaji yatakayoandamana na utafiti huu.

Washiriki watapewa maelezo kamili kuhusu matokea ya utafiti kwa afya yao.

Swali: Kuna faida gani kwa washiriki wa utafiti huu?

Jibu: Wanawake watakaoshiriki watapata uchunguzi wa afya yao bila malipo yoyote. Uchunguzi utakuwa juu ya magonjwa ya zinaa, mwendo na kiasi cha damu mwilini. Kama mshiriki hajapimwa virusi vya ukimwa tutampima bila malipo. Hii itasaidia kujua hali yake ili ajitunze vilivyo. Mshiriki akiwa na virusi tutamwelezea jinsi ya kujitunza pamoja na jamii yake. Washiriki hawa watajulishwa jinsi ya kujiunga na vikundi ambapo wanaweza kupata elimu na mawaidha zaidi. Waadhiriwa watakaokuwa katika kiwango cha kutumia dawa za ukimwi watazipata bila malipo hapa hospitali ya Tigoni. Dawa hizi zimetolewa bila malipo na Chuo Kikuu cha Nairobi (University of Nairobi Institute of Tropical and Infectious Diseases, UNITID). Utafiti huu pia utakuwa na manufaa kwa walioadhiriwa na pia kuwasaidia wengine wajue jinsi ya kujikinga vilivyo kutokana na virusi vya ukimwi.

Swali: Nina haki gani kama Mshiriki kwa utafiti huu?

Jibu: Watafiti watahakikisha kuwa habari yote utakayotoa itawekwa siri. Maandishi yote kuhusu washiriki yatahifadhiwa katika kabati zilizofungwa. Hivyo basi hakuna atakayeweza kupata habari yoyote kukuhusu. Damu na viungo vingine vya utafiti katika maabara vitaandikwa nambari ya mshiriki wala sio majina yake kamili. Kushiriki kwa utafiti huu itakuwa kujitolea na hakuna atakaye adhibiwa kwa kukataa kushiriki.

Swali: Mawaidha kabla ya kupimwa virusi vya ukimwi

Jibu: Kila mtu ana haki ya kujua hali yake ya afya. Hii inasaidia kujikinga na pia kuikinga jamii yake. Habari kuhusu magonjwa ya zinaa, na kuhusu mambo ya uzazi kwa jumla, itasaidia washiriki na pia wenzao wanaohusiana nao. Mtu akishajua hali yake ya afya anatokwa na wasi wasi. Mtu anapojua hali yake mapema huweza kujikinga ili afya yake isidhoofike. Kina mama pia hupata elimu kuhusu jinsi wanavyoweza kumkinga mtoto kabla na pia wakati wa kuzaliwa. Habari zozote kuhusu hali ya mshiriki itawekwa siri na haitajulikana na mtu mwingine pasipo ruhusa ya mshiriki.

Swali: Mawaidha baada ya kupimwa virusi vya ukimwi

jibu. Baadhi ya watu huwa na wakati mgumu hasa wanapogundua kuwa wana virusi vya ukimwi. Hali hii huwafanya wasiweze kuwaeleza wapenzi wao na jamii kwa jumla. Mtafiti mwenye kutoa mawaidha kuhusu mambo ya kijamii atawasaidia washiriki na mbinu za kukubali hali yao na jinsi watakavyoweza kuwaeleza kati ya jamii zao. Kuwatuma wagonjwa kujiunga na vikundi vya watu walioambukizwa virusi vya ukimwi imeonyesha kuwasaidia hasa wanapopitia wakati mgumu. Habari anazopewa mtu mwenye virusi vya ukimwi zitaonyesha jinsi afya yake ilivyoahirika na hatua anazohitaji kuchukua ili kurekebisha hali yake vilivyo. Pia kuna maelezo kuhusu magonjwa mengine yanayohusiana na virusi vya ukimwi, kwa mfano Kifua Kikuu. Pia wagonjwa watakata maelezo kuhusu dawa za ukimwi. Elimu kuhusu jinsi mama aliyeahiriwa anavyoweza kumzuia mtoto anapozaliwa asipate virusi vya ukimwi itatolewa, pamoja na elimu juu ya njia za kupanga uzazi.

Swali: Nikiwa na jambo ningependa kuuliza kuhusu kushiriki kwangu nitamuuliza nani?

Jibu: Utafiti huu umeidhinishwa na kamati kuu yenye kuhusika na utafiti katika chuo kikuu cha Nairobi na pia hospitali kuu ya Kenyatta. Washiriki waweza kuwasiliana na mwenyekiti wa kamati hii kwa simu ifuatayo: **272-3600**.

Watafiti wa chuo kikuu cha Nairobi wako tayari kushughulikia maswali yoyote ambayo washiriki wangependa kujibiwa. Washiriki waweza kuwasiliana na watafiti kwa simu zifuatazo:

Eunice: 0721-727 300 or 0733-875 602

Anne: 0722-398 657

Teresa Tel: 0722-394 190

AMA

Mkurugenzi wa UNITID, Chuo kikuu cha Nairobi.

Prof. B. Estambale Simu: 272-6765

APPENDIX 4

FOMU YA MAKUBALIANO NA MSHIRIKI- (MAKTABA)

- NIMESOMA NA KUYAELEWA MAKSUDI YA UTAFITI HUU, PAMOJA NA JUKUMU LANGU KAMA MSHIRIKI.

AU _____

- NIMEELEZWA NA KUELEWA MAKSUDI YA UTAFITI HUU, NA PIA JUKUMU LANGU KAMA MSHIRIKI.
- NILIPEWA NAFASI YA KUULIZA MASWALI KUHUSU UTAFITI HUU NA NIKAJIBIWA NA WATAFITI.
- NAELEWA KWAMBA KUSHIRIKI KWANGU KATIKA UTAFITI HUU NI KWA HIARI WALA SIKULAZIMISHWA.
- NIKO TAYARI KUPIMWA VIRUSI VYA UKIMWI NA KUFANYIWA UCHUNGUZI MWENGINE WA MAGONJWA YA ZINAA.
- NIMEKUBALI KWAMBA VIUNGO VYANGU VYA UTAFITI VITASAFIRISHWA KATIKA SEHEMU ZINGINE ZA NCHI NA PIA NCHI ZA NG'AMBO KWA UTAFITI ZAIDI NA KWAMBA MAJINA YANGU HAYATAFICHULIWA.
- NIMEKUBALI KUWA DAMU NA VIUNGO VINGINE VYANGU VYA UTAFITI VIHIFADHIWE NA KUTUMIKA KWA UTAFITI.
- NIMEPEWA MAANDISHI YA HABARI KWA MSHIRIKI.

✓ MSHIRIKI (SAHIHI) _____ TAREHE ___/___/___
dd mm yyyy

✓ MTAFITI (SAHIHI) _____ TAREHE ___/___/___
(AMA MWAKILISHI) dd mm yyyy

APPENDIX 5

PATIENT CONTACT INFORMATION

DATE: ____/____/____
 dd mm yyyy

Interviewer _____

MUCOSAL IMMUNITY STUDY (MIS)

MIS STUDY NUMBER:

--	--	--

NATIONAL I.D. NO: _____

LOCATOR INFORMATION

PATIENT'S FULL NAMES (3).....

ADDRESS.....

TELEPHONE CONTACT.....whose?.....

VILLAGE.....

LOCATION.....

CHIEF/ SUB-CHIEF.....

WOMEN'S GROUP.....

WOMEN'S GROUP LEADER.....

CHURCH.....

NEAREST SCHOOL/ SHOPPING CENTER.....

DIRECTIONS TO HER HOME (Sketch detailed map on Page 2)

DETAILED TRACER MAP TO PATIENT'S HOME

PLUGHER IT'S QUESTIONNAIRE

<input type="checkbox"/> YES	<input type="checkbox"/> NO	<input type="checkbox"/> NA	<input type="checkbox"/>
<hr/>			
1. How often do you use the toilet? (YES)	YES	<input type="checkbox"/>	<input type="checkbox"/>
2. How often do you use the toilet? (NO)	NO	<input type="checkbox"/>	<input type="checkbox"/>
3. How often do you use the toilet? (NA)	NA	<input type="checkbox"/>	<input type="checkbox"/>
4. How often do you use the toilet? (YES)	YES	<input type="checkbox"/>	<input type="checkbox"/>
5. How often do you use the toilet? (NO)	NO	<input type="checkbox"/>	<input type="checkbox"/>
6. How often do you use the toilet? (NA)	NA	<input type="checkbox"/>	<input type="checkbox"/>
7. How often do you use the toilet? (YES)	YES	<input type="checkbox"/>	<input type="checkbox"/>
8. How often do you use the toilet? (NO)	NO	<input type="checkbox"/>	<input type="checkbox"/>
9. How often do you use the toilet? (NA)	NA	<input type="checkbox"/>	<input type="checkbox"/>
10. How often do you use the toilet? (YES)	YES	<input type="checkbox"/>	<input type="checkbox"/>
11. How often do you use the toilet? (NO)	NO	<input type="checkbox"/>	<input type="checkbox"/>
12. How often do you use the toilet? (NA)	NA	<input type="checkbox"/>	<input type="checkbox"/>
13. How often do you use the toilet? (YES)	YES	<input type="checkbox"/>	<input type="checkbox"/>
14. How often do you use the toilet? (NO)	NO	<input type="checkbox"/>	<input type="checkbox"/>
15. How often do you use the toilet? (NA)	NA	<input type="checkbox"/>	<input type="checkbox"/>
16. How often do you use the toilet? (YES)	YES	<input type="checkbox"/>	<input type="checkbox"/>
17. How often do you use the toilet? (NO)	NO	<input type="checkbox"/>	<input type="checkbox"/>
18. How often do you use the toilet? (NA)	NA	<input type="checkbox"/>	<input type="checkbox"/>
19. How often do you use the toilet? (YES)	YES	<input type="checkbox"/>	<input type="checkbox"/>
20. How often do you use the toilet? (NO)	NO	<input type="checkbox"/>	<input type="checkbox"/>
21. How often do you use the toilet? (NA)	NA	<input type="checkbox"/>	<input type="checkbox"/>

APPENDIX 6

ELIGIBILITY QUESTIONNAIRE

TDS NO: MISSING: N/A:

Please tick "YES" or "NO"

1. Normal Pap smear result within the last year: YES NO
2. Age between 18-45 years: YES NO
3. Regular menstruation last 2 cycles: YES NO
(Length of cycle to be 25-35 days)
4. Not pregnant (as per urine pregnancy test): YES NO
5. Not currently using hormonal contraception: YES NO
6. Never been exposed to HAART: YES NO
7. Uterus and cervix intact: YES NO
8. No systemic disease the last 3 months (interviewer to explain):
YES NO
9. No delivery or abortion in the last 3 months: YES NO
10. Client in good health: YES NO
11. CD4 Count \geq 250cells/ μ L (to do assay): YES NO
12. Willing to participate: YES NO

NB: QUESTIONS 1, 4 & 11 TO BE COMPLETED AFTER TEST IS DONE.

13. Available to come to the clinic on a weekly basis for two months:

YES NO

14. (To the interviewer): In your assessment the client is mentally competent to give informed consent:

YES NO

If the client answered 'no' to any of the questions above then she is not eligible for the study. Ask her if she knows another woman in her area who could participate in the study to kindly send her to us.

IF CLIENT IS ELIGIBLE BOOK HER FOR THE NEXT APPOINTMENT IN TWO WEEKS (pending lab results for STI screening).

NEXT APPOINTMENT DATE: / /
 dd mm yyyy

MIS STUDY SCREENING SPECIMEN FORM

Date Specimen taken: ____/____/____
 dd mm yyyy

First day of LMP: ____/____/____
 dd mm yyyy

SPECIMENS TAKEN (LABEL- date, initials, MIS no, Visit no, test required):

5.0 ml EDTA Blood: Yes No (CD4 COUNT)
(Purple TOP)

5.0 ml Serum: Yes No (HIV ELISA)
(Red TOP)

5.0 ml Serum: Yes No (SYPH)
(Red TOP)

EXAMINATION ROOM

1st Endocervical swab: Yes No (CT/ GC PCR)

2nd Endocervical swab: Yes No (CT/ GC PCR)

3rd Endocervical brush: Yes No (PAP smear)

1st High vaginal swab (HVS): Yes No (-80°C-store in tube)

2nd HVS: Yes No (*Candida* - KOH in tube)

3rd HVS: Yes No (TV- IN-POUCH CULTURE TUBE)

4th HVS: Yes No (TV/ KOH wet prep-RECORD)

5th HVS: Yes No (pH/ Gram stain, fix methanol-ON SITE)

Vagina pH: _____ (Record immediately -ON SITE).

MIS NUMBER: [][][]

PATIENT INITIALS: [][][]

APPENDIX 7

CONTACT UPDATE FORM

FOLLOW-UP INFORMATION

TELEPHONE CONTACT.....whose?.....

Screening DATE: ___/___/___
 dd mm yyyy

Enrolment DATE: ___/___/___
 dd mm yyyy

- ARE THERE ANY CHANGES IN THE DIRECTIONS TO THE CLIENT'S HOME?

(If yes, Sketch Detailed map on next Page for TRACER ACTION)

MIS NUMBER: [][][]

PATIENT INITIALS: [][][]

10. Number of lifetime partners: [][][]

11. Number of current regular sex partners: [][]

12. Number of sex partners in the last one week: [][]

13. Did you use condoms during the last week? Yes No

14. Why do you use condoms? (multiple answers possible)

i. To prevent HIV/ STDs

ii. I have multiple partners

iii. As a contraceptive

iv. I have no stable partner

v. I don't trust partner(s)

vi. At partner's request

vii. Because I am HIV+

viii. Other _____

15. Who supplies condoms: Self Partner self & partner

16. Why would you stop condom use? Never Expensive to buy

To get pregnant If I Got married Man unwilling No sexual partner

Other _____

17. Why are you not using contraceptives? Want to get pregnant

Have no partner Planning to start I use condoms Side effects

Use other methods Undecided Other _____

MEDICAL HISTORY

18. Had injections in past 1 year: Yes No If yes why _____

19. Blood transfusion in past 1 year: Yes No If yes why _____

20. Admission to hospital in past 1 year: Yes No

If yes why _____

21. Have you had any of the following during the last 2 months?

a) Vaginal discharge: Yes No

b) Ulcer on genitals: Yes No

If yes, please give details of the problem _____

CURRENT MEDICAL PROBLEMS

22. Weight loss: Yes No Days Months

23. Diarrhoea: Yes No Days Months

24. Fever: Yes No Days Months

25. Night sweats: Yes No Days Months

26. Headaches: Yes No Days Months

27. Dizziness: Yes No Days Months

28. Fatigue: Yes No Days Months

29. Swollen glands: Yes No Days Months

30. Cough: Yes No Days Months

31. Itchy Rash: Yes No Days Months

32. Genital ulcer: Yes No Days Months

33. Genital warts: Yes No Days Months

34. Vaginal discharge: Yes No Days Months

35. Dysuria: Yes No Days Months

36. Abdominal pain: Yes No Days Months

37. Vulva itch: Yes No Days Months

38. Chest pain: Yes No Days Months

39. Any other medical problem? _____

DRUGS

40. Have you been on medication(s) the last 6 months? Yes No

If yes type and reason: _____

41. Ever been an IV drug abuser? Yes No

42. Ever been on AntiTB drugs? Yes No

43. Are you on antiretroviral therapy (ARV)? Yes No

HERBAL MEDICINES

44. History of use: Always Occasionally Never

45. If ever used, why _____

46. Has any of the following been a problem? Allergic Rhinitis

Contact dermatitis Asthma

Other allergy-related problems: _____

47. Do you take medication for allergies? Yes No

If yes specify _____

PHYSICAL EXAMINATION:

48. Body weight: [][][] KG

49. Height: [][][] CM

50. Pulse rate: [][][] beats/ min

51. Blood pressure: Systolic [][][] Diastolic [][][] mmHg

EXAMINATION ROOM:

52. Gynaecological examination:

i. Anatomical features of the FGT: Normal Not normal

If not normal, specify _____

ii. Genital inflammation: Yes No

iii. Vaginal discharge: Yes No

If yes describe discharge _____

NB: Give client appointment according to menstrual diary.

8. Body weight:

--	--	--	--

 KG

9. Pulse rate:

--	--	--	--

 Beats/ min

10. Blood pressure: Systolic

--	--	--	--

Diastolic

--	--	--	--

mmHg

EXAMINATION ROOM11. Lower abdominal pain: Yes No 12. Pain on urination: Yes No 13. Genital itching: Yes No 14. Vaginal discharge: Yes No 15. Any other medical problem? _____
_____16. Any current medication (specify) _____

FOLLOW-UP SPECIMENS

RECEPTION ROOM

Specimen Date: ____/____/____
dd / mm / yy

Specimens taken:

5.0 ml EDTA blood: Yes No (repeat CD4 on visit 5 for HIV pos)

5.0 ml serum (RED TOP): Yes No (repeat HIV test on visit 7 for NEG cases)

EXAMINATION ROOM

Vaginal pH: _____ (wet pH strip with HVS, before CVL collection)

1st High vaginal swab: Yes No (for gram stain and pH)

2nd High vaginal swab: Yes No (in transport tube for DNA extraction for microflora characterization)

3rd High vaginal swab: Yes No (in dry transport tube for DNA extraction for microflora characterization/ @-80 °C)

4th High vaginal swab: Yes No (CULTURE- streak onto plate on-site/ insert in transport medium)

10.0 ml CVL:

Yes No (See CVL PROTOCOL overleaf)**PROCESSING FRESHLY COLLECTED CVL****NB: USE GREEN OR ORANGE SAMPLE LABELS.****Before CVL procedure collect the 4 HVS SWABS:****ON SITE TESTS ON CVL-REFER TO INDIVIDUAL SOPs**

1. Prepared a **GRAM STAIN** on the CVL: Yes No
2. Record pH: _____
3. Total pooled **volume of CVL**: _____ **MLS**
4. CVL wet prep for presence of **SPERMATOZOA**: Present Absent
5. Presence of **mucous in CVL**: Yes No
6. CVL **Haemoglobin level** : _____ **(RECORD)**
7. **KEEP 1.0 ML uncentrifuged CVL**: Yes No

MIS NUMBER: [][][]

PATIENT INITIALS: [][][]

DATE: ____/____/____
 dd mm yyyy

INTERVIEWER _____

VISIT NUMBER: []

APPENDIX 12

NAME OF CONSULTING DOCTOR: _____

Reason(s) for visit: _____

Treatment or advice given to client: _____

MIS STUDY PATIENT PAPSMEAR CLINICAL NOTES

MIS NUMBER: [][][] PATIENT INITIALS: [][][]

DATE OF SPECIMEN COLLECTION: ___/___/___
dd mm yyyy

APPENDIX 15

PAP SMEAR LABORATORY REQUEST FORM

DATE OF REQUEST: ___/___/___
dd mm yyyy

REPORT TO BE SENT TO: MIS STUDY (TN KIAMA)

**STAMP
HERE**

Presence of endocervical cells: YES NO

RESULTS (please tick the appropriate box)

1. Normal: YES NO

2. Atypical squamous cells of undetermined significance (ASCUS): YES NO

3. Low grade squamous intraepithelial lesion (LGSIL): YES NO

4. High grade squamous intraepithelial lesion (HGSIL): YES NO

5. Invasive carcinoma: YES NO

6. Not interpretable [repeat sample]: YES NO

REMARKS: _____

NAME OF PATHOLOGIST REPORTING: _____ SIGNATURE: _____ DATE: ___/___/___
dd mm yyyy

DATE: ___/___/___
dd mm yyyy

INTERVIEWER: _____

MIS NUMBER [][][]

PATIENT INITIALS [][][]

APPENDIX 16**APPOINTMENT CARD****CLIENT'S COPY**Visit number: (2, 3, 4, 5, 6, 7 OR 8)

<u>JAN 2008</u>	Mon	Tue	Wed	Thur	Fri	Sat	Sun
		1	2	3	4	5	6
	7	8	9	10	11	12	13
	14	15	16	17	18	19	20
	21	22	23	24	25	26	27
	28	29	30	31			

Tick:

- 3 DAYS OF SEXUAL ABSTINENCE (INTERVIEWER to cross, x, 3 days IN RED and explain)
- START AND END OF PERIODS (CLIENT to circle, 0, all days of bleeding)
- APPOINTMENT DATE (date TCA by INTERVIEWER and mark with a star * IN BLACK)

NB: Please avoid having sex and using vaginal products on the 3 days before the appointment
Q. Whom do I contact if I have any concerns or questions about my participation in the study?

A. This study has been approved by the Ethics and Research Committee of the University of Nairobi and Kenyatta National Hospital. Clients may contact the Chairman of the committee, Tel 2 273 600.

The study team is also available to discuss any concerns arising during the visits to the clinic.

Clients may also contact staff from the Reproductive Health Project, University of Nairobi:

Eunice Tel: 0721-727 300/ 0733-875602

Anne Tel: 0722-398 657

Teresa Tel: 0722-394 190

OR

Reproductive Health Project Leader and Director, UNITID, University of Nairobi.

Prof. B. Estambale Tel: 2726300 Ext. 43163

NB: Interviewer to kindly give the Client this Contact Card.

DATE: / /
dd mm yyyy

INTERVIEWER: _____

MIS NUMBER [][][]

PATIENT INITIALS [][][]

APPOINTMENT CARDFILE COPYVisit number: (2, 3, 4, 5, 6, 7 OR 8)

<small>JAN 2008</small>	Mon	Tue	Wed	Thur	Fri	Sat	Sun
		1	2	3	4	5	6
	7	8	9	10	11	12	13
	14	15	16	17	18	19	20
	21	22	23	24	25	26	27
	28	29	30	31			

Tick:

- 3 DAYS OF SEXUAL ABSTINENCE (INTERVIEWER to cross, ×, 3 days IN RED and explain)
- START AND END OF PERIODS (CLIENT to circle, ⊖, all days of bleeding)
- APPOINTMENT DATE (date TCA by INTERVIEWER and mark with a star * IN BLACK)

NB: Please avoid having sex and using vaginal products on the 3 days before the appointment.

Q. Whom do I contact if I have any concerns or questions about my participation in the study?

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Anne Tel: 0722-398 657

Teresa Tel: 0722-394 190

OR

Reproductive Health Project Leader and Director, UNITID, University of Nairobi.

Prof. B. Estambale Tel: 272-6765

APPENDIX 18

MIS COHORT 777- GRAM STAIN SCORING SHEET

SPECIMEN: HVS

Patient	Visit	Nugent	Ia	Iab	Ib	I-like	II	III	III	0	LAB	Yeast	MOB	STR	STA	BN	LAB-osis	CytVag	Remark	Final Score	
001	1																				
MWK	3																				
	4																				
	5																				
	6																				
	7																				
	8																				
	003	1																			
	MNW	3																			
4																					
5																					
6																					
7																					
8																					
004		1																			
ANG		3																			
	4																				
	5																				
	6																				
	7																				
	8																				
	005	1																			
	KNK	3																			
4																					

APPENDIX 18

APPENDIX 19

BUFFERS FOR EASYMAG DNA EXTRACTION PROCEDURE

1M Tris-HCl 1 l (stock in refrigerator)

- a. Dissolve 121.14 g tris(hydroxymethyl)aminomethane (MW = 121.14) in 800 ml H₂O
- b. Adjust pH to 8.0 with concentrated HCl.
- c. Adjust volume with water to 1L.

Make aliquots in 50 ml-falcons. Autoclave.

- d. Label with your name, content (1 M Tris HCl, pH 8.0) and date of preparation
- e. Store in at 4°C

20 mM Tris-HCl

- a. Transfer 1 ml of a 1 M Tris-HCl solution into a 50 ml Falcon tube.
- b. Adjust volume with water to 50 ml.
- c. Label with your name, content (20mM Tris HCl, pH 8.0) and date of preparation
- d. Store at RT

Mutanolysin/PK buffer

Composition: 20 mM Tris-HCl, pH 8.0, 0.5% SDS

- a. Transfer 9.5 ml of a 20 mM Tris-HCl solution into a 14 ml Falcon tube.
- b. Add 0.5 ml of 10% SDS.
- c. Label with your name, content (20mM Tris HCl 0.5% SDS, pH 8.0) and date of preparation
- d. Store at room temperature

Preparation of Proteinase Kinase (PK) buffer

Composition: 20 mM Tris-HCl and PK

- a. Add 4.0 ml 20 mM Tris-HCl to the PK bottle.
- b. Mix gently and make 100 µl aliquots
- c. store at -20 °C

Preparation of Mutanolysin buffer

- a. Add 400 µl HPLC water to the mutanolysin (Streptomyces globisporus, FicolI Pharmacia, Biotech USA. Sigma-Aldrich) container.
- b. Mix and make 20 µl aliquots.

APPENDIX 20

This protocol was adapted from the method by Baele et al. (2000).

MATERIALS

Reagents

Bacterial cells

Formamide, deionized

Genomic DNA (up to 1 µg)

High Fidelity Mix (1.1X) (Invitrogen)

Oligonucleotides (Welsh & McClelland 1991):

primer 1 (T5A) 5'-AGTCCGGTGCTCTAACCAACTGAG-3' (10 µM)

primer 2 (T3B) 5'-GGTCGCGGGTTCGAATCC-3' (10 µM)

Label 1/5th of primer 2 with a fluorescent dye.

SDS-NaOH lysis buffer

Size standards (labeled with a fluorescent dye) (Applied Biosystems or Beckman Coulter):

- GS-400 High Density size standard and GS-500 size standard (Applied Biosystems) (for use with the ABI Prism 310 Genetic Analyzer)
These standards contain ROX-labeled fragments in the range of 50 to 500 bp.
- LIZ600 size standard (Applied Biosystems) (for use with the ABI Prism 3100 or 3130XL Genetic Analyzers)
LIZ600 contains LIZ-labeled fragments in the range of 40 to 600 bp.
- CEQ-600 size standard (Beckman Coulter) (for use with the CEQ 8000 Genetic Analysis System)
Use with CEQ sample loading solution (SLS) (Beckman Coulter 608082).

Software for data analysis

- Basehopper (www.BaseHopper.be)
- Genescan Analysis software (Applied Biosystems) (for use with ABI Prism only)
- Phylip/Neighbor (<http://evolution.genetics.washington.edu/phylip.html>)
A manual is downloadable from the same website.
- TreeView (<http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>)

Equipment

Capillary electrophoresis apparatus (e.g., ABI-Prism 310, 3100 or 3130XL Genetic Analyzer; Applied Biosystems or CEQ 8000; Beckman Coulter)

Heater

Microcentrifuge

Plates, microtiter

Thermal cycler

METHOD

The same bacterial strain should be used as a reproducibility control with each PCR and capillary electrophoresis run.

DNA Preparation (Alkaline DNA Extraction)

Although tDNA-PCR can, in theory, be used directly on clinical samples for which only one or two species are expected to be present, isolation culture is usually needed to avoid interference of patterns from different strains rendering the obtained pattern uninterpretable. We describe a short alkaline DNA extraction method, as labor-intensive methods (e.g. phenol-chloroform extractions) are generally not necessary.

1. Harvest a 1- μ L loopful of bacterial cells from an agar medium, and suspend in 20 μ L of SDS-NaOH lysis buffer.
2. Heat the cells for 5 minutes at 95 °C. Add 180 μ L of H₂O and centrifuge for 5 minutes at 16000g to pellet debris.
3. Use the supernatant containing the extracted DNA directly in the PCR (Steps 4-5), or store at -20 °C for further use.

PCR

Because capillary electrophoresis is used to separate the amplified tDNA-intergenic spacers, small PCR volumes, as low as 10 μ L, are sufficient.

4. Prepare the PCR using a 10- μ L total volume per sample:
 - 9.1 μ L High Fidelity Mix 1.1X
 - 0.1 μ L primer 1
 - 0.1 μ L primer 2 (of which 0.02 μ L is fluorescently labeled)
 - 0.7 μ L DNA template from Step 3

5. Perform the following cycling program:

No. of Cycles	Temperature	Time
1	94 °C	2 minutes
30	94 °C	30 seconds
	50 °C	1 minute
	72 °C	1 minute
1	72 °C	30 minutes
1	10 °C	hold

Capillary Electrophoresis

Instructions for preparing samples for capillary electrophoresis using an ABI Prism 310 (Step 6), ABI Prism 3100 or 3130XL (Step 7), or CEQ 8000 (Step 8) are provided.

6. To prepare PCR products for analysis using an ABI Prism 310:
 - i. For each PCR product, mix 12 μ L of deionized formamide with 0.3 μ L of GS-400 High Density size standard and 0.2 μ L of GS-500 size standard (which both have ROX-labeled fragments in the range of 50 to 500 bp for use with the ABI Prism 310). Prepare the mixture of formamide and marker in a total volume sufficient for all PCR products, and divide into 12.5 μ L/well in microtiter plates.
 - ii. Add 1 μ L of tDNA-PCR product from Step 5 to each well. Denature the mixtures by heating at 95 °C for 3 minutes. Place the mixtures directly on ice for at least 15 minutes.
7. To prepare PCR products for analysis using an ABI Prism 3100 or 3130XL:
 - i. For each PCR product, mix 8.75 μ L formamide with 0.25 μ L of LIZ600 size standard with LIZ-labeled fragments in the range of 40 to 600 bp. Prepare the mixture of formamide and marker in a total volume sufficient for all PCR products and divide into 9 μ L/well in microtiter plates.
 - ii. Add 1 μ L of tDNA-PCR product from Step 5 to each well. Denature the mixtures by heating at 95°C for 3 minutes. Place the mixtures directly on ice for at least 15 minutes.
8. To prepare PCR products for analysis using a CEQ 8000, mix 0.1 μ L of CEQ-600 size standard with 40 μ L CEQ sample loading buffer (SLS) and 0.5 μ L of tDNA-PCR product.
9. Perform capillary electrophoresis:
 - i. Use the following conditions for electrophoresis using an ABI Prism 310:

Temperature	60 °C
Constant voltage	1.5 kV
Current: approximately	10 mA

Capillary length	47 cm (50- μ m diameter)
Performance Optimized Polymer	4
ii. Use the following conditions for electrophoresis using an ABI Prism 3100 or 3130XL:	
Oven temperature	60 °C
Poly Fill Volume	6500 steps
Current stability	5.0 μ Amps
Prerun voltage	15 kV
Pre run time	180 seconds
Injection voltage	3.0 kV
Injection time	30 seconds
Voltage number of steps	20 nk
Voltage step interval	15 seconds
Data delay time	60 seconds
Run voltage	15 kV
Run time	1500 seconds (depending on the length of the size standard)
Capillary length	36 cm
Performance Optimized Polymer	7
iii. Use the following conditions for electrophoresis using a CEQ 8000, using the recommended Frag-4 separation method:	
Capillary temperature	50 °C
CEQ capillaries	75- μ m internal diameter and 33-cm length
Temperature	90 °C for 120 seconds
Injection voltage	2.0 kV for 30 seconds
Separation voltage	4.8 kV for 60 minutes

Data Analysis

Data analysis using the ABI (Step 10) or CEQ (Step 11) systems is described.

10. To analyze data generated using an ABI system:

i. Normalize the electropherograms using GeneScan Analysis software.

The fragment lengths are derived from the peak positions after interpolation with the peak positions of the size standard fragments.

ii. In GeneScan Analysis, select the sample files for identification. Extract the sample files in table format using the "export tables" menu. Save resulting data as a file on your computer, preferably in C:\Samples or C:\Tables.

See Troubleshooting.

11. To analyze data generated using a CEQ system, use the default fragment analysis parameters and export the results to a comma delimited (csv) file.

See Troubleshooting.

12. Divide the tables into separate sample file tables using the "Divide file" menu in the BaseHopper software.

The digitized fingerprints (sample files) can then be given species names and strain designations.

13. Using Basehopper, compare the sample files to an existing library (database) of other sample files using the Dice, Jaccard or differential base pairs (dbp) algorithm. To generate a library with known fingerprints (libraries), use the BaseHopper manual. *For tDNA-PCR, the dbp algorithm has been shown most useful, because additional peaks that are not reproducible are not taken into account in the similarity calculation, and thus do not influence the similarity percentage between an unknown strain and the library pattern.*

See *Troubleshooting*.

14. For cluster analysis using BaseHopper, calculate the distance matrix using the Dice, Jaccard or differential base pairs (dbp) algorithms. Cluster the samples using Phylip/Neighbor, employing the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) or Neighbor Joining algorithm.

15. Construct a dendrogram using TreeView, which can be opened directly through the BaseHopper menu.

16. [Optional] Represent the fingerprints graphically using the Graph option of BaseHopper.

TROUBLESHOOTING

Problem: There is amplification in the negative PCR control.

[Steps 10-11]

Solution: Due to the manipulation of PCR products in all PCR-based methods, the reactions can become contaminated. If negative controls produce a DNA smear, several steps can be taken to eliminate the contamination:

- Repeat the PCR using fresh reagents.
- Use filtered tips to avoid introduction of contaminants via aerosol from the pipette.
- Physically separate the areas in the laboratory where reactions are set up and processed. Prepare and pipette the PCR mixture at one bench, and then add the DNA to the reaction in a different location in the laboratory. Also, use different pipettes for reaction preparation and pipetting of DNA.
- It is possible to prepare the tDNA-PCR reaction mixture in a large volume and to aliquot large series of 9.3- μ L volumes into tubes or microtiter plates. These can be stored at -80°C . Only the DNA extract must be added when an isolate is ready to be identified. This may reduce the chance of contamination by reducing the number of manipulations.

Note that with techniques like tDNA-PCR, which start from a high concentration of target DNA, contamination is a minor problem. Even when negative controls show some amplification, the contaminating fragments are not observed after amplification of the bacterial DNAs, probably due to competition in favor of the abundantly present target DNA.

Problem: No amplification is observed.

[Steps 10-11]

Solution: Check the quality of the starting DNA. Perform an alternative DNA extraction method if necessary.

The species of a limited number of groups (e.g., corynebacteria) often yield no or a low number of amplicons. tDNA-PCR cannot be used for most species of these groups using the described primer pair. Select other tRNA primers.

Problem: The obtained peak pattern is not present in the database.

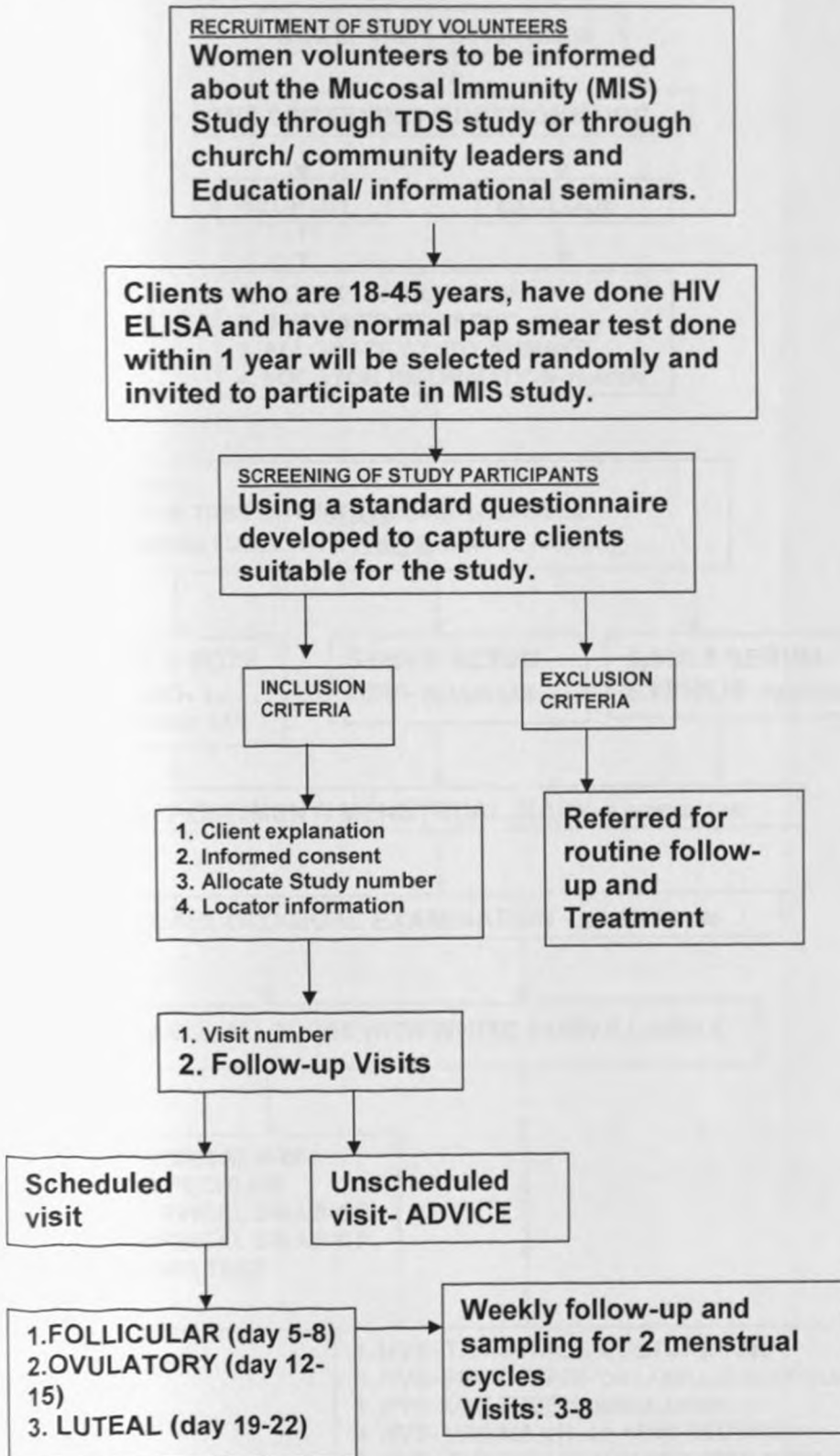
[Step 13]

Solution: Mixed cultures may be present in the DNA sample. Repeat isolation culture of the sample. Alternatively, the species may not yet be in the identification library. Identify the strain by using a reference technique, e.g., 16S rRNA gene sequencing. Add the name of the species and its corresponding fingerprint to the library.

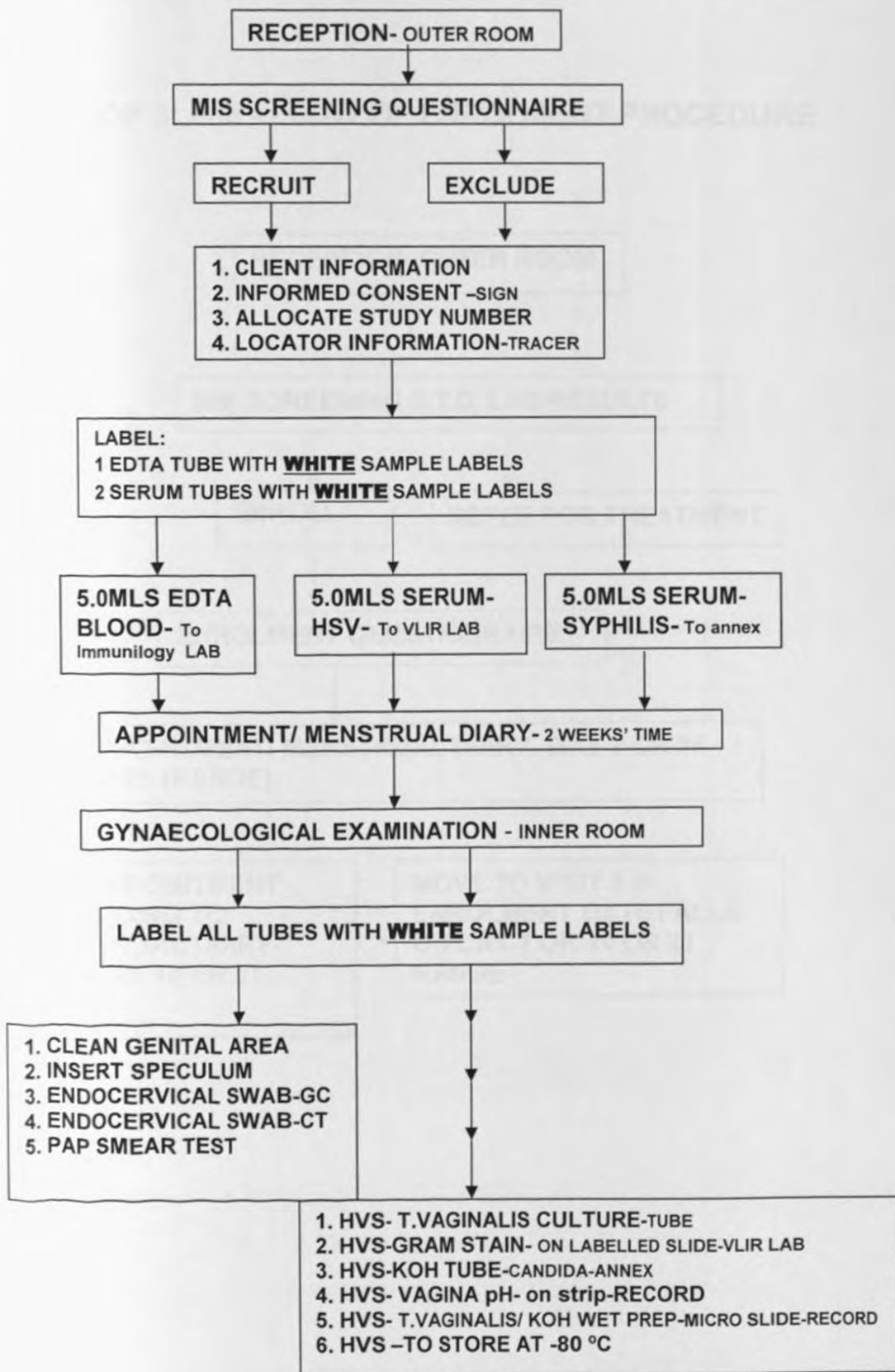
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SOP 1: SUMMARY OF FLOW OF VOLUNTEERS FOR MIS STUDY



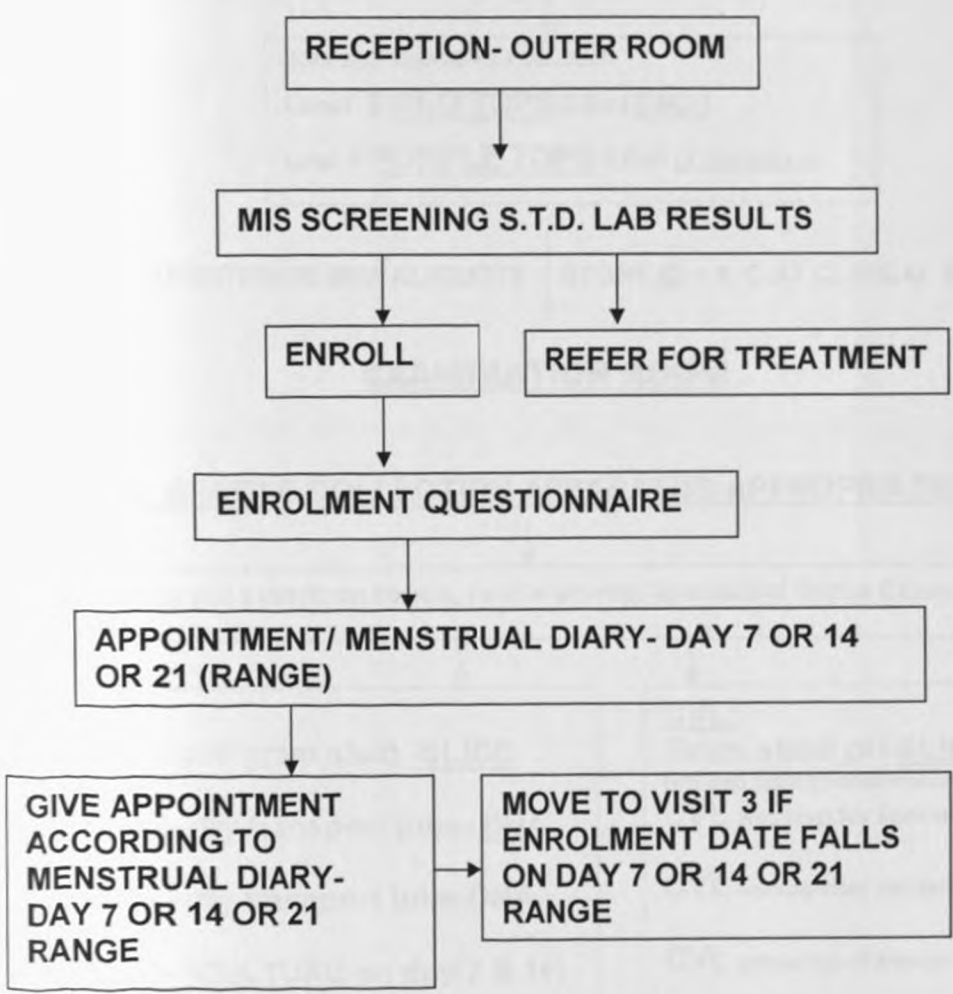
SOP 2: MIS FLOW OF SCREENING PROCEDURES



SOPA FOLLOW-UP VISIT 3-8

QUALITY OF FOLLOW-UP SPECIMEN COLLECTION

SOP 3: MIS - FLOW OF ENROLMENT PROCEDURE



SOP4: FOLLOW-UP VISIT 3-8

SUMMARY OF FOLLOW-UP SPECIMEN COLLECTION

Use Questionnaire for visit #. Take all measurements-wt, ht, BP, pulse.

COLLECT BLOOD AS FOLLOWS:

Label **2 RED TOPS**-5.0ml **EACH**

Label **4 PURPLE TOPS**-5.0ml (**5 ON VISIT 4**)

PROCESS WITHIN 30 MIN/ ALIQUOTS STORE @ + 4 °C AT CLINICAL SITE.

EXAMINATION ROOM

LABEL ALL SAMPLE COLLECTION APPARATUS APPROPRIATELY

Calmly put patient on couch, Feet in stirrup, Speculum/ Gynae Exam

1st HVS- pH/ gram stain -SLIDE

2nd HVS- dry transport tube- DNA

3rd HVS- dry transport tube-DNA

4th HVS- (CULTURE on day 7 & 14)

CVL:

Gram stain/ pH-SLIDE

Record total pooled volume

CVL wet prep for sperms.....

CVL hemoglobin contamination

CVL presence of mucus.....

Centrifuge and process (see CVL PROTOCOL)

TRANSPORT TO LAB WITHIN 3 HOURS OF COLLECTION

AT KNH: 1. SET CULTURE PLATE IN ANAEROBIC JARS.

2. FREEZE CVL. SERUM, PLASMA, HVS @ -80 °C .

3. CONFIRM STORAGE CHARTS FOR ALL SAMPLES + BACK-UPS.

SOP 5: GENERAL INSTRUCTIONS

Universal Precautions

Since a medical history and examination cannot identify all patients with infectious agents, standard precautions should be consistently used for all patients.

The blood, body fluids and other specimens of every patient must be considered to be potentially infectious.

Protective labware must be worn at all times while handling specimens.

SOP 6: SCREENING VISIT SPECIMENS (LABELING & IDENTIFICATION)

- **WHITE LABELS WILL BE USED FOR SCREENING SPECIMENS.**

Scope

This standard operation procedure (SOP) is to be followed by all clinical and laboratory staff involved in MIS study at Tigoni clinical site and Kenyatta National Hospital (KNH) labs.

Applicable to

The Principal Investigator, the Laboratory Technologists and the Nursing staff assigned to the project.

Purpose

Ready-made self adhesive labels (25X19mm) printed with the following details will be used for every specimen collected:

Mucosal Immunity study Date..... Patient Initials..... Study Number..... Visit Number..... Test Required.....

Procedure For Labeling Specimens

- First fill in the details on the label.
- Stick it on the specimen container (serum tube, swab tube, culture tube, Petri dish etc).
- Put the intended specimen.

SCREENING VISIT SPECIMENS (visit 1):

Tests required will be abbreviated as follows:

1. 5.0 ml EDTA blood- **CD4**
2. 5.0 ml serum for syphilis- **SYPH**
3. 5.0 ml serum for herpes simplex virus- **HSV**
4. X2 Neisseria/ Chlamydia endocervical swab- **GC/CT PCR**
5. T. vaginalis culture tube- **TV CULT**
6. Potassium hydroxide candida tube- **KOH**
7. Gram stain- **WRITE DETAILS ON FROST-END SLIDE FOLLOWING LABEL ORDER**

SOP 7: FOLLOW-UP VISIT SPECIMENS (LABELING & IDENTIFICATION)

- **GREEN & ORANGE LABELS WILL BE USED FOR FOLLOW-UP SPECIMENS (VISIT 3-8)**

Scope

This standard operation procedure (SOP) is to be followed by PI, clinical and laboratory staff involved in MIS study at Tigoni clinical site and Kenyatta National Hospital (KNH) labs.

Applicable to

The Principal Investigator, the Laboratory Technologists and the Nursing staff assigned to the project.

Purpose

Ready-made self-adhesive labels (25X19mm) printed with the following details will be used for every specimen collected:

Mucosal Immunity study Date..... Patient Initials..... Study Number..... Visit Number..... Test Required.....

Procedure For Labeling Specimens

- First fill in the details on the label.
- Stick it on the specimen container (serum tube, swab tube, Petri dish etc).
- Put the intended specimen.

Follow-up tests required will be abbreviated as follows:

1. Cervicovaginal Lavage- **CVL**
2. EDTA Blood- **PLASMA**
3. Serum (CRP ELISA)- **SERUM**
4. Gram stain- **WRITE DETAILS ON SLIDE FOLLOWING LABEL ORDER**
5. 2X High vaginal swab - **DNA**
6. Culture plate- **STICK COMPLETED LABEL ON PETRI DISH**

SOP 8: TRANSPORT & STORAGE OF SPECIMENS

ALL SPECIMENS WILL BE ACCOMPANIED BY A FILLED-IN LAB REQUEST FORM

1. Samples will be aliquoted at the site of collection and kept at +4 to +8 °C.
2. Cool Specimens will be transported in a cool box on ice packs.
3. In the lab storage charts will be prepared and specimens transferred to -80 °C.

TABLE OF TRANSPORT & STORAGE CONDITIONS

SPECIMEN	TEST	TRANSPORT TEMP.	STORAGE TEMP.	COMMENTS
EDTA BLOOD	CD4 COUNT	ROOM TEMP (RT)	Processed same day	Ensure all specimens are labeled appropriately.
ENDOCERVICAL	PAPSMEAR	ROOM TEMP (RT)	ROOM TEMP (RT)	Ensure cap tightly closed.
FIXED SLIDES	GRAMSTAIN	SLIDE BOX @ (RT)	ROOM TEMP (RT)	
TV POUCH	TV	ROOM TEMP (RT)	CULTURE @ 37°C	
KOH TUBE/SWAB	CANDIDA	ROOM TEMP (RT)		hold upright in rack
EDTA BLOOD	ALIQUOTS	4 °C /COLD PACKS	MINUS 80 °C	
SERUM	ALIQUOTS	4 °C /COLD PACKS	MINUS 80 °C	
CVL	ALIQUOTS	4 °C /COLD PACKS	MINUS 80 °C	
HVS	DNA	4 °C /COLD PACKS	MINUS 20 °C	

CLINICAL PROCEDURES

SOP 9: CHECKING BLOOD PRESSURE

1. OBJECTIVE

This SOP describes the procedure to be followed when checking the blood pressure of clients at Tigoni clinical site.

2. SCOPE

This procedure is applicable to trained clinical staff.

3. DEFINITIONS

MmHg-Millimeters of mercury

4. BACKGROUND

Blood pressure is checked to help recognize any changes in the systolic and diastolic arterial pressure of a patient.

Blood pressure varies relative to heart's pumping of blood. The heart should be thought of as a dual pump, with a right and left side. **Systole** represents the active pumping of blood from the **ventricles** into the circulation. The right side of the heart takes venous/returning blood from the body and sends it to the lungs to be oxygenated (**Pulmonary circulation**). Measuring blood pressure on the right side of the heart requires very complicated equipment. The left side of the heart takes the oxygenated blood and sends it to the rest of the body. When you take blood pressure reading, you measure pressure in the **systemic circulation**.

The highest pressures occur during **systole** as blood is ejected into the **aorta** and subsequent arteries of the body. **Diastole**, the heart's resting phase, follows systole. During diastole the ventricles fill with more blood. Systemic pressure falls until more blood is ejected during systole.

The blood pressure cuff applies external pressure to a circumferential ring around the upper arm. When the pressure is great enough, it forcibly closes the main artery of the upper arm – the **brachial artery**.

5. PROCEDURE

5.1 Requirements:

A tray with the following:

- i. Sphygmomanometer.
- ii. Stethoscope.
- iii. Patient file to record the reading.
- iv. Pen

5.2 Preparation.

5.2.1 Equipment.

- i. Arrange the observation tray with the above mentioned equipments.
- ii. Ensure the sphygmomanometer is in good working order.

5.2.2 Stethoscope.

- i. Check the tubing for holes.
- ii. Clean any wax from the ear tips. Point ear pieces forward.
- iii. Use the bell portion of the stethoscope to listen for the sounds.
- iv. Be sure to place it lightly over the artery, with skin contact all around.

5.2.3 Cuff

- i. Make sure the cuff size suits your patient.
- ii. Check to see that the screw valve on the ball works properly.
- iii. Pump the bladder and watch for any air leaks. If the mercury column or aneroid needle does not rise steadily as you pump the ball, suspect a leak.

5.2.4 Sphygmomanometer

- i. Check that the needle is at zero mark at the start and the end of the measurements.
- ii. Place the manometer in your direct line of sight.

5.2.5 Subject

- i. Explain the procedure to the patient.
- ii. Determine whether your patient/participant has smoke or drunk alcohol within the last 15 minutes. Both can alter the readings.
- iii. If possible ask her to sit or lie down for at least 5 minutes.
- iv. If a patient comes in Running, breathless or worried then she should rest for 15 minutes before you take her blood pressure again.
- v. She should be lying down with one or two pillows or sitting on a chair with the arm resting on a table at the same level with the heart.
- vi. Remove all clothing from the arm. Avoid using the arm which has an I.V line, shunts, edema, injury or paralysis. Do not place the cuff over clothing or let roll-up sleeve constrict arm.
- vii. Ask your patient not to talk during the measurements.

5.2.6 Environment.

There should be enough space with good ventilation and good light.

5.3 Method

- i. Apply the cuff on the upper arm of the patient.

- ii. Place the sphygmomanometer on the bed/locker or table on the same level with the patient's heart.
- iii. Connect the cuff and the sphygmomanometer.
- iv. Locate the bronchial artery at the front of the elbow joint by placing your fingers of your left hand on the inner side of the elbow.
- v. Pump air into the cuff to let the mercury rise until pulsation is no longer felt by the fingers or heard when the stethoscope is applied over the artery.
- vi. Apply the stethoscope to both ears and place the trumpet end over the bronchial artery.
- vii. Release the pressure slowly and as the mercury falls listen to the first beat, i.e, the systolic pressure-noting the mercury reading on the sphygmomanometer.
- viii. As the pressure falls the beat changes and the nurse notes the reading at which the reading changes or stops, i.e. the diastolic pressure.
- ix. Remove the stethoscope from the ears.
- x. Release all the pressure from the cuff-remove the apparatus by untying the cuff and disconnect the tubing's from the machine.
- xi. Fold the cuff nicely and put it neatly inside the machine and lock it.
- xii. Record the readings on the patient's file.
- xiii. Explain the findings to the patient/ client and reassure.
- xiv. Note and report any abnormalities.
- xv. Normal range of Blood pressure is 100/60 to 140/90 mmHg. Average blood pressure is 120/80mm Hg. This varies with age, sex and also the patient's condition.

5.4 Clearing

- i. Take the tray back to the duty room or put it back on the table for the next patient/participant.
- ii. Keep the sphygmomanometer and the stethoscope in their required place.

5.5 Interpret results

In adults, hypertension is defined as regular BP readings >140/90 mmHg. **6.**

REFERENCES

The human Heart - www.worldinvisible.com/apologet/humbody/heart.htm
 Procedure manual for Nurses-second edition: July 1998

SOP 10: CHECKING BODY WEIGHT

OBJECTIVE

To measure the body weight of all MIS clients.

SCOPE

This SOP is to be followed by the Principal investigator and the nurses attached to the project.

PROCEDURE

1. Inform the client that you wish to take her weight.
2. Instruct the patient to disrobe (only heavy outer garments).
3. Request the patient to remove their shoes.
4. Ask the patient to step on the weighing machine and remain still.
5. Record the weight in kilogrammes in the space provided in the patient's file.

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SOP 11: CHECKING THE PULSE RATE

LEARNING OBJECTIVES

1. Instruct the client to relax while seated upright in a comfortable chair.
2. With the forearm supported on the table palpate the pulsations of the Radial artery just proximal to the wrist joint.
3. Having localized the pulsation do a count of consecutive pulsations over a period of one minute.
4. Record the result in beats/minute in the space provided in the patient's file.

PROCEDURE

1. Explain the procedure to the client and ensure they are relaxed and seated upright in a comfortable chair.

2. Support the client's forearm on the table and palpate the radial artery just proximal to the wrist joint.

3. Once the pulse is localized, count the number of consecutive pulsations over a period of one minute.

4. Record the result in beats/minute in the space provided in the patient's file.

ASSESSMENT

The client should be able to describe the procedure and the location of the radial artery.

The client should be able to palpate the radial artery and count the number of consecutive pulsations over a period of one minute.

The client should be able to record the result in beats/minute in the space provided in the patient's file.

SOP 12a: PHLEBOTOMY

OVERVIEW

(EDTA -Purple tops; SERUM -Red tops)

Blood collected in EDTA tubes will be used for the complete blood cell count and CD₄ cell count and some cytokine assays. EDTA is a more stable anticoagulant, compared to heparin, which has been shown to induce apoptosis (125). Using a 'vacutainer' tube EDTA blood and serum will be drawn consecutively. The clot in serum will be removed by centrifugation and the serum will be transferred into labelled plastic vials for storage and subsequent assays.

PROCEDURE for EDTA BLOOD:

1. Collect blood using EDTA as an anticoagulant.

NB: Heparin and citrate are not recommended for use as anticoagulants.

2. Centrifuge for 15min @ 3,000 rpm within 30 min of collection.

NB: Grossly haemolysed samples are not suitable for measurement of IL-1.

3. Make aliquots of 0.5ml, put in labeled sealable bags and leave in the fridge.

4. Prepare sample log sheet and freeze at -80 °C in the lab.

PROCEDURE for SERUM:

1. Collect serum in a plain serum tube (RED TOP).

2. Allow samples to clot for 30 min (Timer).

3. Centrifuge for 15min @ 3,000 rpm within 30 min of collection.

4. Make aliquots of 0.5ml cryovials, put in labeled sealable bags and leave in the fridge.

5. Prepare sample log sheet and freeze at -80 °C in the lab.

SOP 12b: PHLEBOTOMY

PROCEDURES

1. OBJECTIVE

This Standard Operating Procedure (SOP) describes the procedure to be followed when performing phlebotomy at the clinical site.

It describes the process of performing a venepuncture including;

- i. Proper subject identification procedures.
- ii. Proper equipment selection and use.
- iii. Order of draw for multiple tube phlebotomy.
- iv. Preferred venous access sites, factors to consider in site selection and ability to differentiate between the feel of a vein, tendon and artery.
- v. Subject care following completion of venepuncture.
- vi. Safety and infection control procedures.
- vii. Quality assurance.

2. SCOPE :

All clinical staff in conducting VLIR/RH studies.

3. DEFINITIONS

Standard Operating Procedures (SOPs): Detailed, written instructions to achieve uniformity of the performance of a specific function.

Subject: An individual who participates in a study.

CRF – Case Report Form: A printed, optical, or electronic document designed to record all of the protocol-required information to be reported to the PI/ sponsor on each study subject.

Source Documents: Original documents, data, and records (e.g., hospital records, clinical and office charts, laboratory notes, memoranda, subjects' diaries or evaluation checklists, pharmacy dispensing records etc.

Phlebotomy is the act of drawing or removing blood from the circulatory system through a cut (incision) or puncture in order to obtain a blood sample for analysis and diagnosis.

Phlebotomist is the person who performs the phlebotomy procedure.

IV: Intravenous

SST: Serum Separation Tube

PST: Plasma Separation Tube

EDTA: ethylenediaminetetraacetic acid

4. BACKGROUND

Phlebotomy is performed by a nurse or a technician known as a phlebotomist.

Phlebotomy is carried out for various reasons;

- i. Sample collection for laboratory tests.
- ii. Blood donation.
- iii. Treatment of blood disorders.

When studies are conducted and phlebotomy procedures are part of the study requirement; the subject has the right to:

- i. Considerate, respectful care.
- ii. Confidentiality of all communications and other records pertaining to the subject's health.
- iii. Expect reasonable safety practices and safe environment.
- iv. Know the identity and professional status of individuals providing service and to know which study doctor or other practitioner is primarily responsible.
- v. Obtain from the practitioner complete and current information about diagnosis, treatment, and any known prognosis, in terms the subject can reasonably be expected to understand.
- vi. Reasonable informed participation in decisions involving the subject. The subject has the right to refuse participation in such activity.

5. PROCEDURES

5.1 Subject relations and identification

The phlebotomist should be professional, courteous, and understanding in all contacts with the subject.

- i. Greet the subject and identify yourself and indicate the procedure that will take place. Effective communication - both verbal and nonverbal - is essential.
- ii. Proper subject identification is MANDATORY.
- iii. If possible, speak with the subject during the process. The subject who is at ease will be less focused on the procedure.
- iv. Always thank the subject and excuse yourself courteously when finished.

5.2 Equipment

The following are needed for venipuncture:

- i. Evacuated Collection Tubes - The tubes are designed to fill with a predetermined volume of blood by vacuum. The rubber stoppers are color coded according to the additive that the tube contains. Various sizes are available. Blood should **NEVER** be poured from one tube to another since the tubes can have different additives or coatings.

- ii. Needles - The gauge number indicates the bore size: the larger the gauge number, the smaller the needle bore. Needles are available for evacuated systems and for use with a syringe, single draw or butterfly system.
- iii. Holder/Adapter - use with the evacuated collection system.
- iv. Tourniquet - Wipe off with alcohol and replace frequently.
- v. Alcohol Wipes - 70% isopropyl alcohol.
- vi. Povidone-iodine wipes/swabs - Used if blood culture is to be drawn.
- vii. Gauze sponges - for application on the site from which the needle is withdrawn.
- viii. Adhesive bandages / tape - protects the venipuncture site after collection.
- ix. Needle disposal unit - needles should NEVER be broken, bent, or recapped. Needles should be placed in a proper disposal unit IMMEDIATELY after their use.
- x. Gloves - can be made of latex, rubber, vinyl, etc.; worn to protect the subject and the phlebotomist.
- xi. Syringes - may be used in place of the evacuated collection tube for special circumstances.

5.3 Venipuncture site selection

Although the larger and fuller median cubital and cephalic veins of the arm are used most frequently, wrist and hand veins are also acceptable for venipuncture.

Certain areas are to be avoided when choosing a site:

- 1) Extensive scars from burns and surgery - it is difficult to puncture the scar tissue and obtain a specimen.
- 2) The upper extremity on the side of a previous mastectomy - test results may be affected because of lymphoedema.
- 3) Hematoma - may cause erroneous test results. If another site is not available, collect the specimen distal to the hematoma.
- 4) Intravenous therapy (IV) / blood transfusions - fluid may dilute the specimen, so collect from the opposite arm if possible. Otherwise, satisfactory samples may be drawn below the IV by following these procedures:
 - i. Turn off the IV for at least 2 minutes before venipuncture.
 - ii. Apply the tourniquet below the IV site. Select a vein other than the one with the IV.
 - iii. Perform the venipuncture. Draw 5 ml of blood and discard before drawing the specimen tubes for testing.
- 5) Cannula/fistula/heparin lock - blood should not be drawn from an arm with a fistula or cannula without consulting the attending study doctor.
- 6) Edematous extremities - tissue fluid accumulation alters test results.

5.4 Procedure for vein selection

- i. Palpate and trace the path of veins with the index finger. Arteries pulsate, are most elastic, and have a thick wall. Thrombosed veins lack resilience, feel cord-like, and roll easily.
- ii. If superficial veins are not readily apparent, you can force blood into the vein by massaging the arm from wrist to elbow, tap the site with index and second finger, apply a warm, damp washcloth to the site for 5 minutes, or lower the extremity over the bedside to allow the veins to fill.

5.5 Order of drawing blood

Blood collection tubes must be drawn in a specific order to avoid cross-contamination of additives between tubes. The recommended order of draw is:

- 1) First - blood culture tube (yellow-black stopper)

2) Second - non-additive tube (red stopper or SST)

3) Third - coagulation tube (light blue stopper). If just a routine coagulation assay is the only test ordered, then a single light blue stopper tube may be drawn. If there is a concern regarding contamination by tissue fluids or thromboplastins, then one may draw a non-additive tube first, and then the light blue stopper tube.

- 4) Last draw - additive tubes in this order:

- i. SST (red-gray, or gold, stopper). Contains a gel separator and clot activator.

- ii. Sodium heparin (dark green stopper)

- iii. PST (light green stopper). Contains lithium heparin anticoagulant and a gel separator.

iv. EDTA (lavender stopper)

- v. ACDA or ACDB (pale yellow stopper). Contains acid citrate dextrose.

- vi. Oxalate/fluoride (light gray stopper)

NOTE: Tubes with additives must be thoroughly mixed. Erroneous test results may be obtained when the blood is not thoroughly mixed with the additive.

5.6 Performing a venipuncture

- i. Approach the subject in a friendly, calm manner. Provide for their comfort as much as possible, and gain the subject's cooperation.
- ii. Identify the subject correctly.
- iii. Properly fill out appropriate information in the specimen collection forms in the patient's file and the lab request form indicating the test(s) ordered.
- iv. Label all appropriate tubes. **Instruction on SOP 2 & SOP 3.**

- v. Position the subject. The subject should sit on a chair, lie down or sit up in bed. Hyperextend the subject's arm.
- vi. Select the venipuncture site.
- vii. Apply the tourniquet 3-4 inches above the selected puncture site. Do not place too tightly or leave on more than 2 minutes.
- viii. The subject should make a fist without pumping the hand.
- ix. Prepare the subject's arm using alcohol prep. Cleanse in a circular fashion, beginning at the site and working outward. Allow to air dry.
- x. Grasp the subject's arm firmly using your thumb to draw the skin taut and anchor the vein. The needle should form a 15 to 30 degree angle with the surface of the arm. Swiftly insert the needle through the skin and into the lumen of the vein. Avoid trauma and excessive probing.
- xi. When the last tube to be drawn is filling, remove the tourniquet.
- xii. Remove the needle from the subject's arm using a swift backward motion.
- xiii. Press down on the gauze once the needle is out of the arm, applying adequate pressure to avoid formation of a hematoma.
- xiv. Dispose of contaminated materials/supplies in designated containers.
- xv. Process specimens promptly according to **SOP 4**.

5.6.1 If an incomplete collection or no blood is obtained

- i. Change the position of the needle. Move it forward (it may not be in the lumen)
- ii. Or move it backward (it may have penetrated too far).
- iii. Adjust the angle (the bevel may be against the vein wall).
- iv. Loosen the tourniquet. It may be obstructing blood flow.
- v. Try another tube. There may be no vacuum in the one being used.
- vi. Re-anchor the vein. Veins sometimes roll away from the point of the needle and puncture site.

5.6.2 If blood stops flowing into the tube:

The vein may have collapsed; re-secure the tourniquet to increase venous filling. If this is not successful, remove the needle, take care of the puncture site, and redraw.

The needle may have pulled out of the vein when switching tubes. Hold equipment firmly and place fingers against subject's arm, using the flange for leverage when withdrawing and inserting tubes.

5.6.2 Problems other than an incomplete collection:

5.6.2.1 Haematoma

A hematoma forms under the skin adjacent to the puncture site - release the tourniquet immediately and withdraw the needle. Apply firm pressure.

5.6.2.2 To prevent a hematoma

- i. Puncture only the uppermost wall of the vein.
- ii. Remove the tourniquet before removing the needle.
- iii. Use the major superficial veins.
- iv. Make sure the needle fully penetrates the upper most wall of the vein. (Partial penetration may allow blood to leak into the soft tissue surrounding the vein by way of the needle bevel)
- v. Apply pressure to the venipuncture site.

5.6.3 Punctured artery

The blood is bright red (arterial) rather than venous. Apply firm pressure for more than 5 minutes. Select another site and draw with consent from the subject.

5.7 To prevent hemolysis (which can interfere with many tests):

- i. Mix tubes with anticoagulant additives gently 5-10 times.
- ii. Avoid drawing blood from a hematoma.
- iii. Avoid drawing the plunger back too forcefully, if using a needle and syringe, and avoid frothing of the sample.
- iv. Make sure the venipuncture site is dry.
- v. Avoid a probing, traumatic venipuncture.

6. REFERENCES

- i. Messinezy, Maria and T. C. Pearson. "Polycythaemia, Primary (Essential) Thrombocythaemia and Myelofibrosis (ABC of Clinical Haematology)." *British Medical Journal* 314 (22 February 1997): 587–90.
- ii. Wolfe, Yun Lee. "Case of the Ceaseless Fatigue." *Prevention Magazine* (July 1997): 88–94.
- iii. <http://medstat.med.utah.edu/WebPath/TUTORIAL/PHLEB/PHLEB.html>

SOP 13: GYNAECOLOGICAL EXAMINATION

1. OBJECTIVE

This Standard Operating Procedure (SOP) describes the procedures for subjects undergoing a gynecological examination.

2. DEFINITIONS

Endocervical swab; The endocervical swab is used to collect a specimen from the endocervix to test for *Neisseria gonorrhoea* and *Chlamydia trachomatis*. These infections commonly affect the endocervix. The most common tests done are microscopic examination, culture and/ or Elisa. These tests enable accurate diagnosis as many women with these infections are either asymptomatic or have non specific symptoms.

High vaginal swab; The high vaginal swab is taken from the upper part of the vagina using a swab. This specimen is used to test for *Candida albicans*, *Trichomonas vaginalis* and bacterial vaginosis. The most common tests done are microscopic examination (wet prep) and/or culture.

Pap smear; the Pap smear is taken to test for cervical precancer and cancer.

3. SCOPE

This procedure is applicable to all clinical staff of VLIR/IRH.

4. BACKGROUND

This examination is performed to elicit findings in the female reproductive system.

A gynaecological examination involves checking of the external genitalia and pelvic organs and has 3 main components: external genital examination, speculum examination and bimanual examination. A gynaecological examination is also referred to as vaginal examination.

The purpose of this examination is to;

- a. Establish or confirm diagnosis.
- b. Resolve ambiguities.
- c. Alert the clinical personnel on pathological findings suggesting gynecological malignancy.
- d. Facilitate appropriate management of each patient including referral.
- e. Collect gynecological specimens.

The samples that can be collected when carrying out a gynaecological examination include; Pap smear, high vaginal swab and endocervical swab.

5. PROCEDURE

5.1 Equipment and Materials

- i. Speculum (bivalve speculum e.g. Cusco's)
- ii. Examination couch
- iii. Light source
- iv. Gloves (disposable or high level disinfected)
- v. Cotton
- vi. Swabs
- vii. Gauze
- viii. Small container with (warm) water to lubricate speculum
- ix. Microscope slides, specimen containers, culture media, Petri dishes and request forms
- x. Antiseptic solution
- xi. Labels
- xii. Patient record/ file
- xiii. Clean water and soap for washing hands
- xiv. 0.5% chlorine solution (prepared daily) for decontamination
- xv. Trash bin
- xvi. Screen

5.2 Client Assessment

1. Greet the subject and introduce yourself to create rapport.
2. Explain why the examination is necessary and describe the procedure to be done. Explanation should be simple and precise.
3. Discuss the potential benefits and risks of the examination.
4. Take a reproductive health history or, if medical record is available, confirm the following information
 - i. Age
 - ii. Parity (number births, living children, abortions/miscarriages)
 - iii. Currently pregnant
 - iv. Previous and current contraception
 - v. First day of last menstrual period (LMP) and menstrual cycle (interval between menstrual periods in days, regular/irregular)
 - vi. Age at first intercourse
 - vii. Number of sexual partners
 - viii. History of STDs, including HIV/AIDS

- ix. History of abnormal Pap smear with dates and if any treatment was provided for the same.
- x. Mother or sister(s) had breast or ovarian cancer.
- xi. Any gynaecological operations done.
- xii. Medical and surgical history including any medications and drug allergies.

5.3 PREPARATION

1. Check the gynaecological examination set, (including gloves, swabs, brushes slides & specimen vials, if sample collection is necessary).
2. Ensure the room is well ventilated and clean.
3. Inform the subject of what is going to be done & encourage her to ask questions.
4. Ask subject to empty her bladder before the examination.
5. Ensure privacy then ask her to undress from waist down.
6. Help her on to a clean examination table and position for examination.

5.4 Steps/Tasks

1. Put on examination gloves on both hands.
2. Arrange instruments and supplies on a clean tray.

5.4.1 Abdominal Examination:

1. Ask her to lie on her back (supine position).
2. Inspect abdomen for any skin changes, scars, irregularities, and swelling.
3. Palpate for any masses, noting position, size and attachment to underlying structures. Note any tenderness and muscle guarding.
4. Palpate for enlarged liver and/or spleen.
5. Feel for enlarged inguinal lymph nodes noting size, consistency and tenderness.

5.4.2 Examination of external genitalia

Using gloved hand gently examine for redness, changes in colour, lumps, swelling, unusual discharge, sores, tears and scars around the genitals and in between the skin folds of the vulva.

5.4.3 Speculum Examination

1. Select speculum of appropriate size and shape.
2. Lubricate and warm speculum with normal saline.
3. Placing 2 fingers at the vaginal introitus or just inside gently separate the labia to visualize the introitus.

4. Use the other hand holding the speculum blades together to slip the speculum obliquely into the vagina. When inserting the speculum avoid the sensitive anterior wall and urethra by exerting slight pressure to the posterior vaginal wall. As the speculum is inserted, gently rotate the blades to horizontal position. Gently open the blades so that the cervix can be completely visualized. Be careful not to press on the urethra or clitoris as this is painful. Tighten the screw on the speculum or lock the speculum in the open position
5. Using a good light examine the cervix for any abnormalities such as discharge, bleeding, ulcers, warts etc
6. Collect all the necessary samples.
7. After closing the blades slightly, inspect the vaginal mucosa whilst withdrawing the speculum slowly by gently turning the speculum several degrees in both directions.
8. Remove the speculum and place in 0.5% chlorine solution (1:6) for ten minutes for decontamination

5.4.2 Bimanual examination

1. Separate the labia's with left hand, gently insert the index and middle finger of the right hand into the vaginal canal.
2. Check the cervix:
 - a. Os is open or closed,
 - b. Direction of cervix,
 - c. Irregularities of the surface,
 - d. Consistency,
 - e. Tenderness on movement (cervical exaltation);
- f. When examining the cervix in/before labour check the position, cervical dilation, consistency, effacement and station of the baby.
- g. When examining the cervix with suspicion of/confirmed cancer determine the size of the tumor and possible spread to the vaginal fornices, the parametria, the pelvic walls, the bladder and the rectum. Involvement of the rectum and rectovaginal septum is determined by rectovaginal examination.
3. Placing the other hand on the lower abdomen, palpate the uterus between both hands. Check for position (anteverted, retroverted, mid position), size, surface regularity, consistency and mobility of the uterus.
4. Examine the lateral vaginal fornices and pouch of Douglas for tenderness and any adnexal mass.
5. To detect a utero-/vesico-/recto-vaginal prolapse ask the woman to bear down or cough and inspect if something bulges out of the vagina
6. Inspect & palpate Bartholin's & Skene's glands.

7. Inform subject you are through with the procedure. Assist the subject sit up, get down from couch and get dressed.
8. Thank the subject.

5.4.3 Post Examination Tasks

1. Before removing gloves, dispose of used swabs by placing in a leak proof plastic trash bin
2. Immerse speculum set into 0.5% chlorine solution
3. Remove gloves by turning inside out
4. Wash hands thoroughly with soap and water and dry with clean dry cloth/paper napkins or air dry
5. Put on clean gloves to wipe/clean examination couch with disinfectant after each client
6. Discuss the findings with the subject and answer any questions.
7. Record findings clearly in subject's record illustrate using diagrams if possible

6. REFERENCES

WHO Regional Publication Pg 46 – 48

Procedure Manual for Nurses 2nd Edition July 1998 Pg 278 – 280

Kenya National Reproductive Health Instruction Manual for Service Providers March 2006

Comprehensive cervical cancer control: A guide to essential practice. World Health Organization 2006.

SOP 14: COLLECTION OF ENDOCERVICAL SMEAR

1. OBJECTIVE

This Standard Operating Procedure (SOP) describes how to collect an endocervical swab.

2. SCOPE

This procedure is applicable to all clinical staff of VLIR/RH.

3. DEFINITIONS

- i. **Dyspareunia:** painful intercourse.
- ii. **Dysuria:** difficult or painful urination.
- iii. **HVS** – High vaginal swab
- iv. **Lower genital tract infection:** includes vaginal and cervical infection.
- v. **Pathogen:** a microorganism, such as a bacterium, that lives on and feeds from a host and causes disease.
- vi. **PBS** - Phosphate Buffered Saline
- vii. **Posterior vaginal fornix:** top of the vagina, behind the uterine cervix. Vaginal discharge pools here.
- viii. **RTI** – reproductive tract infection
- ix. **Screening:** examination of usually symptom-free individuals to detect those with signs of a given disease.
- x. **Signs:** abnormalities indicative of disease identified by health care provider on examination of the patient.
- xi. **STI** – Sexually transmitted infections
- xii. **Swab:** a rolled piece of cotton or gauze attached to the end of a stick or clamp, used for applying medications or collecting biological samples from a surface.
- xiii. **Symptom:** abnormal phenomenon experienced by patient and indicative of disease.
- xiv. **Upper genital tract infection:** includes infection of endometrium, fallopian tubes, ovaries and surrounding tissues.

4. BACKGROUND

The endocervical swab is for aetiological diagnosis of cervicitis, diagnosis of Neisseria gonorrhoea and Chlamydia trachomatis infection in women or diagnosis of other pathogens causing RTI's. **The specimen is collected from the endocervix.** Diagnosis is made by microscopic examination, PCR and/or culture.

Both Neisseria gonorrhoea and Chlamydia trachomatis infect the endocervical canal, urethra and bartholins gland. Symptoms will be referable to these

structures: pain with passing urine, painful intercourse, irregular PV bleeding, discharge, etc. It must be noted that many women have unspecific symptoms or no symptoms thus diagnosis through culture/PCR additional tests on the endocervical swab can make the diagnosis become more accurate.

The purpose of these tests is to establish or confirm a diagnosis.

5. PROCEDURE

5.1 Equipment and Materials

- i. Speculum set
- ii. Gloves
- iii. Sterile swabs
- iv. Gauze
- v. Microscope slides, specimen media, Petri dishes
- vi. PBS
- vii. Labels and Patient file.

5.2 Preparation

For client assessment, preparation, speculum examination and post examination tasks, please refer to **SOP (Gynaecological Examination)**

5.3 Specimen collection

1. In case of any discharge around the endocervix wipe it off with gauze or with a swab, then **discard that swab**.
2. Insert appropriate swab into endocervix until most of the tip is not visible. Rotate swab with **firm** pressure against the walls of the endocervix 3-4 times.
3. Place swab in **labeled transport tube** or bottle provided.
Break off the shaft of swab (raise swab well off the bottom of tube or bottle before snapping shaft).
Cap tube or bottle tightly.
4. Transport swabs with the appropriate lab request forms completely filled out.

7. REFERENCES

WHO Regional Publications, Eastern Mediterranean series No. 8 (1995):
Specimen collection and transport for microbiological investigation. Alexandria,
1994, pp 46 – 48

Nursing council of Kenya (1998), Procedure Manual for Nurses 2nd Edition July,
1998, pp 278 – 280.

Management guidelines for sexually transmitted and other reproductive tract
infections. World Health Organization 2005.

SOP 15: COLLECTION OF PAPSMEAR SPECIMENS

1. OBJECTIVE

This Standard Operating Procedure describes how to collect a Pap smear for the identification of cervical precancerous lesions.

2. SCOPE

This procedure is applicable to all clinical staff of VLIR/RH project.

3. DEFINITIONS

Adenocarcinoma: These are cancer showing gland like characteristics such as cancer arising from the columnar epithelium of the cervical canal.

Atypical squamous cells (ASC): A cytology result of ASC is used to describe "cellular abnormalities that were more marked than those attributable to reactive changes but that fell short of a definitive diagnosis of 'squamous intraepithelial lesion' (SIL)" (2001 Bethesda System of nomenclature). The risk of cancer is very low (0.1–0.2%) and the risk of CIN 2/3+ for any individual patient is also low (6.4–11.9%).

Atypical squamous cells of unknown significance (ASC-US). Options for evaluation include immediate colposcopy, triage to colposcopy by HPV DNA testing, or repeat cytology tests at 6 and 12 months.

Atypical squamous cells, cannot exclude HSIL (ASC-H). This terminology is used to alert clinicians that the risk of CIN 3 or cancer exceeds that of ASC findings but lacks the certainty required for the patient to be given an HSIL interpretation, which would prompt an excision if colposcopic evaluation was negative. Colposcopy is appropriate.

Adenocarcinoma in situ. (AIS) Precursor/ precancer lesions arising from columnar cells of the endocervix.

Bethesda Classification: System of reporting cervical cytology result. Aimed to produce effective communication of cervical cytology result from laboratory to clinician. System includes descriptive diagnosis and evaluation of specimen adequacy. The Bethesda system was last revised in 2001.

Carcinoma in situ CIS: Precancer stage of cancer. Histological abnormality involves the entire thickness of cervical epithelium but does not penetrate the basement membrane of the cervical epithelium.

CIN Classification: This system grades severity of precancer cervical lesions based on histology (microscopic examination of biopsy specimen)

Endocervical curettage (ECC). ECC is performed by scraping the endocervical canal with a designated instrument to obtain a sample of cells from inside the cervical canal.

Endometrial cells: Cells lining the uterine cavity.

Human Papilloma virus (HPV). A common virus that can be sexually transmitted. There are over 120 types of HPV; about 30 involve the genital tract. HPVs are classified as low risk types and high risk types/oncogenic. In some women high risk types of HPV (about 15 HPV types) slowly cause cellular changes in the cervical cells which can over time lead to cervical cancer. In this document, use of the term HPV refers to the high-risk types of the virus unless otherwise noted. The lifetime cumulative risk of getting HPV infection is at least 80%. The vast majority of women clear the virus spontaneously or suppress it to levels not associated with CIN 2/3+. However, HPV is necessary for the development and maintenance of CIN 3. Persistent high-risk HPV is a necessary but not sufficient condition for the development of almost all invasive cancers.

High grade squamous epithelial lesion (HSIL): Term used in Bethesda classification to describe a high grade cervical intraepithelial abnormality. This finding needs to be further investigated and if confirmed needs to be treated as the lesion has a high likelihood of progressing to cervical cancer.

Low grade squamous intraepithelial lesion (LSIL) Term used in Bethesda classification system and describes mild abnormality of cervical epithelium.

Pap : Papanicolaou.

Pre-cancer lesions of cervix: These lesions also referred to as precursor lesion of the cervix, precedes invasive lesions (cancer) of the cervix. One of the most common types of cervical cancer is referred to as squamous cell carcinoma). Precancer lesions are diagnosed on histology e.g. examination of a biopsy specimen taken from the cervix and is based on abnormal characteristics of cervical squamous epithelial cells and is referred to as cervical intraepithelial neoplasia (CIN). CIN is graded as CIN1, CIN 2 and CIN 3 depending upon the proportion of the thickness of the squamous epithelium showing abnormal cells.

Screening: This is a public health intervention used on a population at risk, or target population. Screening is not undertaken to diagnose a disease, but to identify individuals with a high probability of having or of developing a disease in this case cervical cancer. Women targeted for cervical cancer screening may actually feel perfectly healthy and may see no reason to visit a health facility.

Squamous intraepithelial lesion (SIL): These are abnormalities of the squamous epithelium of the cervix considered to be precancer/ precursor lesions . Bethesda Classification distinguishes between low grade SIL (LSIL) and high grade SIL (HSIL). The 2001 Bethesda classification is used for reporting cervical cytology (Pap smear)

Squamous cell carcinoma of the cervix: Cancer arising from the squamous epithelium of the cervix. Most common cancer of the cervix.

4. BACKGROUND

Pap smear is a screening test used to find abnormal cell changes that arise from cancer of the cervix or its precursor lesions (pre-cancer lesions i.e. before cervical cancer develops).

The Pap smear involves collection of a sample of cells from the uterine cervix using a spatula or brush, smeared onto a slide and examined under a microscope for abnormal cells (pre-cancer or cancer). Devices used for pap smear sampling are wooden spatula (Ayre's spatula), endocervical brush or plastic brush/broom. When a Pap smear shows abnormal result it is referred to as positive. Most women with a positive Pap smear need more tests to confirm the diagnosis and to determine the diagnosis and whether treatment is needed.

5. PROCEDURE

5.1 Equipment and Materials

- i. Speculum (high level disinfected)
- ii. Plastic Endocervical brush
- iii. Gloves (disposable or high level disinfected)
- iv. Labels, request forms, patient's study file
- v. Fixative solution (70% ethanol)
- vi. Sterile gauze on sponge holding forceps
- vii. Small container for saline to lubricate the speculum
- viii. Light source
- ix. Soap and water for washing hands
- x. Examination table/ couch
- xi. 0.5% chlorine solution for decontamination

5.2 Preparation

Note: Menstruating women: It is best not to take a Pap smear from women who are actively menstruating or have symptoms of acute infection. Slight bleeding is acceptable.

- i Greet the subject and identify yourself.
- ii Proper subject identification is MANDATORY. Using the MIS patient FILE, confirm that all required information has been provided by the subject.
- iii Explain the procedure, what the test results mean, and why it is important to return for the test result and act on them appropriately. Ensure that the woman has understood and get verbal informed consent for the procedure.
- iv. Prepare the patient for a gynecological examination, and perform a speculum examination. Refer to **SOP 14 (Gynaecological Examination)** for client assessment, preparation, speculum examination and post examination tasks.

- v. Make sure the whole cervix can be seen and the patient is comfortable.

5.3 Pap smear collection

1. Label the Papspin Collection Fluid bottle with patient details (white labels).
2. Examine the cervix and note any abnormalities such as erosion, inflammation, discharge or masses. Record findings on "Clinical Notes" of patient.
3. Insert the longer, central part of the broom into the cervical os and brush the shorter, outer part against the ectocervix.
4. While maintaining gentle pressure in the direction of the cervix, rotate the brush **5 times in either direction**.
6. Immediately remove the white bristle end of the PAPETTE brush from its handle and drop into the vial of Papspin Collection Fluid.
7. Cap the vial tightly.
8. Shake the vial and prepare it for transport.
9. Explain to the woman that the procedure is complete and she can get dressed. Ask the woman if she has any questions.
10. Inform her as to when she should return for results of the test. Emphasize the importance of returning for test results.

5.4 Transporting slides to lab for processing, reading and reporting

Transport Papspin Collection Fluid and brush at room temperature with the appropriate lab form completely filled out.

5.5 Documentation

Document findings in patients' record and on the pap smear Source document file provided by MIS PI.

Common problems with specimen collection or handling

- Too much blood or mucus on swab.
- Failure to adequately clean exocervix.
- Failure to obtain columnar epithelial cells on the swab.
- Touching vaginal surfaces when withdrawing swab.
- Use of any swab other than those included with collection kit.
- Delay in transporting specimen to the laboratory or other improper handling of the transport tube, i.e. failing to properly seal the tube.

REFERENCES

WHO Regional Publications, Eastern Mediterranean series No. 8 (1995): Specimen collection and transport for microbiological investigation. Alexandria, 1994, pp 46 – 48

Nursing council of Kenya (1998), Procedure Manual for Nurses 2nd Edition July, 1998, pp 278 – 280

Comprehensive cervical cancer control: A guide to essential practice. *World Health Organization 2006*.

Alliance for Cervical Cancer Prevention (ACCP), *Planning and Implementing Cervical Cancer Prevention and Control Programs: A Manual for Managers*, Seattle ACCP 2004

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SOP 16: COLLECTION OF HIGH VAGINAL SWAB (HVS)

HVS swabs will be used for the following purposes:

- i) Gram-stain
- ii) Culture of vaginal microflora and T. vaginalis
- iii) TRFLP (DNA isolation, in dry sterile tube)
- iv) For storage (DNA isolation, in dry sterile tube)
- v) T. vaginalis/ Candida wet prep
- vi) pH of vagina

1. OBJECTIVES

This Standard Operating Procedure (SOP) describes how to perform and collect specimen from vaginal fornices for analysis.

2. SCOPE

This procedure is applicable to all clinical staff of VLIR/RH.

3. DEFINITIONS

- i. **BV – bacterial vaginosis.** Bacterial vaginosis is characterized by a shift in vaginal flora. BV can be diagnosed based on clinical (Amsel criteria and Whiff test) or laboratory criteria (Nugent score on gram stain of HVS shows presence of clue cells, reduced or absent lactobacilli, mixed flora of gardnerella-like and anaerobic bacteria).
- ii. **Clue cells:** vaginal cells covered with bacteria; commonly present in women who have a vaginal infection.
- iii. **Dyspareunia:** painful intercourse.
- iv. **Dysuria:** difficult or painful urination.
- v. **HVS – High vaginal swab**
- vi. **Lower genital tract infection:** includes vaginal and cervical infection.
- vii. **Pathogen:** a microorganism, such as a bacterium, that lives on and feeds from a host and causes disease.
- viii. **Posterior vaginal fornix:** top of the vagina, behind the uterine cervix. Vaginal discharge pools here.
- ix. **RTI – reproductive tract infection**
- x. **Screening:** examination of usually symptom-free individuals to detect those with signs of a given disease.
- xi. **Signs:** abnormalities indicative of disease identified by health care provider on examination of the patient.
- xii. **STI – Sexually transmitted infections**

xiii. **Swab:** a rolled piece of cotton or gauze attached to the end of a stick or clamp, used for applying medications or collecting biological samples from a surface.

xiv. **Symptom:** abnormal phenomenon experienced by patient and indicative of disease.

xv. **Upper genital tract infection:** includes infection of endometrium, fallopian tubes, ovaries and surrounding tissues.

xvi. **Whiff test:** Typical fishy smell in BV after adding a drop of 10% KOH to vaginal secretions.

4. BACKGROUND

High vaginal swab (HVS) is the collection of a sample from the posterior vaginal fornix and is taken for aetiological diagnosis of lower genital tract infections, in particular vaginitis, caused by *Candida albicans* or *Trichomonas vaginalis* and bacterial vaginosis.

5. PROCEDURE

5.1 Equipment and Materials

- i. Speculum set
- ii. Gloves
- iii. Sterile swabs
- iv. Frosted slides, specimen containers (for example 2 ml microtube).
- v. Patient file
- vi. Lab Request forms
- vi. Labels
- vii. Normal saline

5.2 Preparation

For client assessment, preparation, speculum examination and post examination tasks, please refer to **SOP 14 (Gynaecological Examination)**

5.3 Specimen Collection

1. Visualize the cervix with a speculum examination. Using a sterile swab, wipe the posterior fornix of the vagina to collect any secretions or discharge.

2. With a non-lubricated speculum in place the cotton swabs will be inserted consecutively into the vaginal vault. Rotate the swab **three times** against the vaginal wall at the mid-portion of the vault and carefully remove to avoid contamination with the vulva and introitus microflora.

3. For each specified test with HVS see individual SOP:

- Gram stain (SOP 17).
- Culture of vaginal microflora (SOP 23).
- Culture of *T. vaginalis* (SOP 22).
- DNA isolation (SOP 24).
- *T. vaginalis* wet prep (SOP 21).
- *Candida* wet prep (SOP 19).
- pH of vagina (SOP 18).

4. Complete the appropriate lab request forms.

5. Transport specimens to the respective labs at the stated conditions (SOP 9).

6. REFERENCES

WHO Regional Publications, Eastern Mediterranean series No. 8 (1995):
Specimen collection and transport for microbiological investigation. Alexandria, 1994, pp 46 – 48

Nursing council of Kenya (1998), Procedure Manual for Nurses 2nd Edition July, 1998, pp 278 – 280

Management guidelines for Sexually transmitted and other reproductive tract infections. World Health Organization 2005.

SOP 17: PREPARATION OF GRAM STAIN (HVS)

FIX SLIDE IN 60% METHANOL, AIR DRY AND PLACE IN SLIDE BOX

Gram Stain Specimen Collection

1. Visualize the cervix with a speculum examination. Using a sterile swab, wipe the posterior fornix of the vagina to collect any secretions or discharge.
2. With a non-lubricated speculum in place insert the cotton swab into the vaginal vault above the cervix.
Rotate the swab **three times** against the vaginal wall at the mid-portion of the vault.
3. Carefully remove the swab to avoid contamination with the vulva and introitus microflora.
4. For **Gram stain** roll the swab onto a **labelled, frosted slide** in a manner that will yield a thin, uniform smear.
Do not spread the material over the slide but roll back and forth several times in the centre of the slide.
5. Leave the smear to air dry.
6. **Methanol fix the slide by flooding with absolute methanol for 1-2 min and rinse with tap water before staining.**
7. Place in slide box for transport to the lab.
8. **FOR PROPER FIXATION STORE ABSOLUTE METHANOL IN A BROWN SCREW-CAPPED BOTTLE AND REPLENISH THE WORKING SUPPLY EVERY TWO WEEKS.**

NB: Keep CVL and HVS swab Gram stain SLIDES IN SEPARATE LABELLED SLIDE BOXES.

SOP 18: DETERMINATION OF VAGINA PH

The pH of the vagina will be determined directly.

Take the **pH of the vagina** by streaking a HVS onto a Color-pHast indicator strip and reading it against the standard provided after 30 seconds.

Record the pH immediately.

Procedure for determination vaginal pH

1. Take a HVS
1. smear onto ph paper.
3. Leave for 60 sec
4. Read against the provided standard
5. Record on patient sample collection form.

SOP 19: WET PREP FOR CANDIDA (HVS)

Principle:

Wet mount is used for the examination of specimens for fungal elements and yeast. The potassium hydroxide will digest proteinaceous components of cells leaving the yeast and fungus intact.

Vaginal candidiasis: *Candida albicans* is a commensal in the mouth, nose, throat, intestine, vagina and skin.

Vaginal candidiasis will be diagnosed microscopically from a vaginal swab in the wet preparation with **10% potassium hydroxide** to detect the presence of yeast cells. Budding yeast cells and filaments can be seen under the microscope. In the Gram stain large gram-positive yeast cells can be observed.

PROCEDURE:

1. Place a drop of saline onto a clean labelled slide.
2. Spread (HVS) swab contents onto the drop and mix well by rolling both sides at the centre of the slide.
3. To make identification of yeast cells easier in wet mount slides, mix the vaginal swab in another drop of 10% potassium hydroxide to dissolve other cells.
4. Cover with a cover slip.
5. Examine at 400X magnification to look for yeast cells and trichomonads.

Results:

1. The mount is positive if budding yeast or spores are seen (YES).
2. If no fungal elements are seen the mount is negative (NO).
3. Record the result in the space provided in the MIS patient's file.

SOP 20: KOH CANDIDA (FOR LAB TEST)

Quality Control:

The QC for the wet mount will be performed in the lab by someone different from the one who performed the wet mount at the clinical site.

PROCEDURE:

1. Label a clean test tube with patient details.
2. Place 1.0ml 10% potassium hydroxide into the tube.
3. Place the HVS swab into the fluid and mix the contents well by swirling the swab around in the fluid.
4. Place the tube with the swab inside in an upright position in the test tube rack.
5. Transport to the lab at room temperature.

SOP 21: WET PREP FOR T. VAGINALIS (HVS)

Microscopy

Plain slides, cover slips, normal saline, HVS, Microscope, Record sheet, pen.

To make a saline wet preparation the specimen will be suspended by rolling the high vaginal swab in a drop of saline on a labeled frosted slide, covered with a cover slip and examined at 100X magnification. Look for typical jerky movement of motile trichomonads.

PROCEDURE:

1. Place a drop of saline onto a clean labelled frosted slide.
2. Spread (HVS) swab contents onto the drop and mix well by rolling both sides at the centre of the slide.
3. Cover with a cover slip.
4. Examine at 400X magnification to look for yeast cells and trichomonads.

Results:

1. The mount is positive if budding yeast or spores are seen (YES).
2. If no fungal elements are seen the mount is negative (NO).
3. Record the result in the space provided in the MIS patient's file.

SOP 22: T. VAGINALIS IN-POUCH CULTURE

Trichomonas vaginalis is a pathogenic flagellate that infects the urogenital tracts of males and females. It is primarily a sexually transmitted disease. Infection in females can result in vaginitis, cervicitis, and urethritis. In males, the most common symptomatic presentation is urethritis. Diagnosis is by microscopy and culture.

Specimen collection

1. With the speculum in place insert the cotton swab and **rotate it three times** against the vaginal wall at the mid-portion of the vault **in either direction**.
2. Remove carefully to avoid contamination with the vulva and introitus microflora.

Culture

T. vaginalis is anaerobic. Without touching the swab against anything, the swab will then be inserted into the In-Pouch TV (Biomed diagnostics Inc, San Jose, CA) culture medium compartment and transported to the laboratory for anaerobic incubation at 37 °C for 5-7 days for the detection of motile trichomonads.

Principle

Bio Med's In Pouch TV (*Trichomonas vaginalis*) Culture System comprises a clear, plastic envelope containing culture medium. The medium is selective for the transport and growth of *T. vaginalis*, while inhibiting the growth of contaminating microorganisms that might interfere with a reliable result. The medium is inoculated with the vaginal fluid sample. The sample is incubated in the envelope, and the envelope is placed directly on the Microscope for reading. Transport and off-site testing can be performed easily due to the flexible packaging and design of the In-Pouch.

Requirements

Materials:

- In Pouch device
- Swabs
- Gloves

Specimen collection and handling

Specimen collection

1. To avoid fluid leakage, move the culture medium at the top of the In Pouch downwards to the middle of the upper chamber.
2. Tear off plastic above white closure.
3. Obtain specimen on a cotton swab. The investigator is instructed to insert the swab in the vagina and **rotate the swab in a circular motion, 3 times clockwise and 3 times counter-clockwise.**
4. Insert swab into upper chamber medium and elute the specimen by rubbing swab between the In-Pouch walls.
5. Remove the swab and discard.
6. Squeeze the closure strip with the thumb and forefinger of one hand.
7. Hold the bottom of the pouch with the other hand and move the medium from the top chamber to the lower chamber by pulling it upwards across the edge of the counter/table in a "shoe shine" motion.
8. Now roll the empty upper chamber down to the top of the label. Fold the tabs over to prevent the In Pouch from reopening.
9. Label and store the In Pouch for transport to the laboratory.

Specimen handling

Once inoculated and sealed, the In Pouch TV medium can be kept up to 48 hours at room temperature if necessary. After 48 hours, incubation at 37°C is required.

Procedures:

- Specimens are received from the field at the end of each day (approximately 2.00 pm).
- Incubate at 37°C on receipt of the specimen.

Interpretation of results

- If trichomonads are seen on any of the microscopic exams, the results are recorded as positive and the specimen is discarded.
- If no trichomonads are seen on initial exams, continue to incubate and re-examine according to schedule above.
- If **NO** trichomonads are visualized after **96 hours (4 days)** and incubation at 37°C, record result as negative and discard specimen.

Limitations

- *T.vaginalis* is a very sticky organism and will adhere to plastic containers, plastic tubes, plastic pipettes, Dacron swabs, condoms, and mucous.
- Use **only cotton swabs**, wire microbiological loops, glass containers, glass tubes and glass pipettes.

References

- Package insert.
- Standard operating procedures on microscopic examination of *Trichomonas vaginalis* from UNIM and CDC-KEMRI clinical diagnostics laboratory.

SOP 23: BACTERIA CULTURE (HVS)

For all MIS study clients, culture of a HVS will be done at day 7 and 14 of each of the two menstrual cycles.

The culture plate (Petri dishes) with medium [tryptic soy agar supplemented with 5 % sheep blood (Becton Dickinson, Franklin Lakes, NJ)] will be prepared in the microbiology lab and transported to the clinical site.

Procedure for Specimen collection and Culture

Obtain a HVS specimen as described previously (SOP 16).

Fix specimen label at an appropriate place on the plate.

1. Visualize the cervix with a speculum examination. Using a sterile swab, wipe the posterior fornix of the vagina to collect any secretions or discharge.
2. With a non-lubricated speculum in place insert the cotton swab into the vaginal vault above the cervix.

Rotate the swab **three times** against the vaginal wall at the mid-portion of the vault.

3. Carefully remove the swab to avoid contamination with the vulva and introitus microflora.

4. Roll the swab onto the culture plate (i.e. Streak the contents of the HVS onto the culture medium) **as quickly as possible** and immediately cover it to **to avoid contamination**.

5. Leave at room temperature and transport to the lab at room temperature for incubation (anaerobic) at 37 °C for 4 days in the microbiology laboratory. All the isolates with different colony morphology will be selected for identification.

The Procedure for culture in the Microbiology lab is explained under microbiology methods.

SOP 24: HVS FOR DNA ISOLATION

For all MIS study clients a HVS for DNA isolation will be obtained at EVERY FOLLOW-UP VISIT of each of the two menstrual cycles.

The DNA HVS will be obtained using a **special swab with a transport tube**.

The swab with specimen will be **transported inside its tube** and stored at -80 °C or at fridge temperature if DNA is to be isolated immediately.

Procedure for Specimen collection and Transport

Obtain TWO HVS specimen as described previously (SOP 16).

Fix specimen labels at an appropriate place on TWO transport tubes.

1. Visualize the cervix with a speculum examination. Using a sterile swab, wipe the posterior fornix of the vagina to collect any secretions or discharge.
2. With a non-lubricated speculum in place **insert the sterile cotton swab from the transport tube** into the vaginal vault above the cervix.
*Rotate the swab **three times** against the vaginal wall at the mid-portion of the vault.*
3. Carefully remove the swab to avoid contamination with the vulva and introitus microflora.
4. **Without touching anything to avoid contamination** carefully return the swab into the transport tube.

5. REPEAT THE ABOVE PROCEDURE TO OBTAIN TWO HVS SWABS PER CLIENT PER VISIT.

6. Leave the swabs in tube at +4°C (fridge temperature) and transport to the lab at +4°C in the specimen carrier.
7. Keep the swabs in the fridge for short storage and at -80 °C for longer storage.
8. Keep the **2nd swab at -80 °C** as back-up storage.

The Procedure for DNA isolation in PCR the lab is explained under microbiology methods.

SOP 25: PREGNANCY DETERMINATION TEST

Principle of the Procedure

The hCG one step pregnancy test is a rapid Chromatographic immunoassay for the qualitative detection Human chorionic gonadotropin in urine to aid the early detection of pregnancy.

Human chorionic gonadotropin is a glycoprotein hormone produced by the developing placenta shortly after fertilization. In normal pregnancy hCG can be detected in urine and serum as early as 7 to 10 days after conception.

In urine specimens the test has a sensitivity of 25 mIU/ mL. The test utilizes a combination of monoclonal and polyclonal antibodies to selectively detect elevated levels of hCG in urine. The test uses two lines to indicate results.

The assay is conducted by immersing the test strip in a urine specimen and observing the formation of coloured lines. The specimen migrates via capillary action along the membrane to react with the coloured conjugate. Positive specimens react with the specific antibody-hCG-coloured conjugate to form a coloured line at the test line region of the membrane. Absence of this colored line suggests a negative result.

To serve as a procedural control, a colored line will always appear in the control line region indicating that proper volume of specimen has been added and membrane wicking has occurred.

Procedure for Use

1. Collect a urine specimen in a clean and dry container.
2. Allow the test, urine specimens and/ or controls to reach room temperature (15-30 °C) prior to testing.
3. With the arrows pointing towards the urine specimen, immerse the test strip vertically in the urine specimen for at least 10-15 seconds.

Do not pass the MAX line on the test strip when immersing the strip.

4. Place the test strip on a clean microscope slide, start the timer and wait for the colored line(s) to appear. Read the result in 3 minutes. Record results in patient file.

Interpretation of Results

POSITIVE: Two distinct colored lines appear. One line should be in the control line region (C) and another line should be in the test line region (T).

NEGATIVE: One colored line appears in the control line region (C). No apparent colored line appears in the test line region (T).

INVALID: Control line fails to appear. Insufficient specimen volume or incorrect procedural techniques are the most likely reasons for control line failure.

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CLOSING QUOTE

***I waited patiently for the Lord;
He turned to me and heard my cry.
He lifted me out of the slimy pit,
out of the mud and mire
He set my feet on a rock
and gave me a firm place to stand.
He put a new song in my mouth
a hymn of praise to our God,
Many will see and fear
And put their trust in the Lord.***

(Psalms 40:1-3, The Holy Bible. New International Version. 1984.
International Bible Society)