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Teresa N. Kiama

Rita Verhelst

Paul M. Mbugua

Mario Vaneechoutte

Hans Verstraelen

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Authors

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Full Length Research Paper

Characterisation of the vaginal microflora of human immunodeficiency virus (HIV) positive and negative women in a sub-urban population of Kenya

Teresa N. Kiama^{1*}, Rita Verhelst², Paul M. Mbugua¹, Mario Vaneechoutte³, Hans Verstraelen², Benson Estambale⁴ and Marleen Temmerman²

¹Department of Medical Physiology, University of Nairobi, P.O. Box 30197-00100, Nairobi, Kenya.

²Department of Obstetrics and Gynaecology, Faculty of Medicine and Health Sciences, Ghent University hospital, Pintelaan 85, Ghent, Belgium.

³Laboratory for Bacteriology Research, Department of Clinical Chemistry, Microbiology, and Immunology, De Pintelaan 85, Blok A, Ghent University, Ghent, Belgium.

⁴Institute of Tropical and Infectious Diseases, University of Nairobi, P.O. Box 30197-00100, Nairobi, Kenya.

Accepted 26 August, 2013

Lactobacilli predominate normal vaginal microflora and are important in maintenance of vaginal health. The current study set out to identify and compare culture isolates of vaginal microflora of human immunodeficiency virus (HIV) positive (HIV⁺) and HIV negative (HIV⁻) women at different phases during menstrual cycle from a sub-urban population of Kenya. Seventy four (74) women, 41 HIV⁺ and 33 HIV⁻, followed up two consecutive menstrual cycles, had high vaginal swabs taken to prepare Gram stains for six visits and anaerobic cultures for four. All 751 isolates identified by t-DNA polymerase chain reaction (PCR) belong to 51 species. Species cultured more frequently in HIV⁺ participants were: *Lactobacillus jensenii* (p=0.01), *Lactobacillus iners* (p=0.02), *Gardnerella vaginalis* (p=0.01) and *Peptoniphilus lacrimalis* (p=0.01). Species cultured more frequently in HIV⁻ women were *Dialister micraerophilus* (p=0.02) and *Streptococcus agalactiae* (p=0.04). *Lactobacillus* predominating both groups were *Lactobacilli crispatus*, *L. jensenii*, *L. iners* and *Lactobacilli vaginalis*. Bacterial vaginosis (BV) was equally high in HIV⁺ and HIV⁻ women. *Lactobacillus* and BV-associated species were cultured more frequently in HIV⁺ women. Minor species differences were found. Predominant *Lactobacillus* in culture were *L. crispatus*, *L. iners*, *L. jensenii* and *L. vaginalis*. These women had lower concentrations of lactobacilli in vaginal microflora than observed in previous studies of Caucasian women.

Key words: Vaginal microflora, human immunodeficiency virus (HIV), menstrual cycle, t-DNA polymerase chain reaction (PCR), culture, bacterial vaginosis.

INTRODUCTION

Perturbation of the healthy vaginal ecosystem, also by loss of hydrogen peroxide (H₂O₂)-producing

*Corresponding author: E-mail: kiamatn777jes@hotmail.com. Tel: +254-717857852/+254-20-4442309.

Abbreviations: BV, Bacterial vaginosis; STIs, sexually transmitted infections; HIV, human immunodeficiency virus; ART, antiretroviral therapy; pap, papanicolaou; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate.

referred to as bacterial vaginosis (BV), is characterized by lactobacilli and massive overgrowth of *Gardnerella vaginalis* and other Gram positive and negative anaerobic bacteria (Marrazzo, 2004). Of concern are recent observations that BV predisposes to acquisition of sexually transmitted infections (STIs), which raises the risk of mother-to-child transmission of human immunodeficiency virus (HIV). Diminished colonisation resistance during BV renders women vulnerable to various infections such as *Trichomonas vaginalis* and *Neisseria gonorrhoeae* (Martin et al., 1999; Wiesenfeld et al., 2003), *Chlamydia trachomatis* (Wiesenfeld et al., 2003), genital herpes simplex (Kaul et al., 2007), human papillomavirus (Watts et al., 2006) and HIV-1 (Spear et al., 2007; Atashili et al., 2008). Further, it is documented that BV propagates replication and vaginal shedding of the HIV-1 (Sha et al., 2005) and HSV-2 viruses (Cherpes et al., 2005), thus enhancing the spread of STIs. Increased mother-to-child transmission of HIV as a consequence of BV has been documented in a prospective study of 463 HIV-1-infected mothers and infants in Kenya (Farquhar et al., 2010). This study found women with BV to be three times more likely to transmit HIV *in utero*. High prevalence of BV in sub-Saharan Africa (Sewankambo et al., 1997; Taha et al., 1998; Paxton et al., 1998; Martin et al., 1999; van de Wijgert et al., 2000; Demba et al., 2005; Morison et al., 2005; Bukusi et al., 2006; McClelland et al., 2009) has therefore become an issue of global concern. It is estimated, for instance, that in endemic areas, nearly one-third of new HIV cases could be prevented if existing BV were cured (Schwebke, 2005). Tackling the HIV burden through eradicating BV and restoring normal vaginal microflora is currently considered part of the most promising answers to the HIV pandemic (Martin et al., 1999; Shin and Kaul, 2008; Bolton et al., 2008).

The last two decades have seen a significant increase in the volume of research focusing on BV and composition of vaginal microflora (Verhelst et al., 2005; Kalra et al., 2007; Srinivasan et al., 2010; Verstraelen et al., 2010). The prevalence of BV varies widely in different populations. It tends to be high in African women, with rates up to 44% having been documented in a Kenyan cohort and 50% in rural Uganda (Paxton et al., 1998; Bukusi et al., 2006). Studies found African American women to have more than two-fold higher prevalence of BV compared to Caucasian American women. The cause of this disparity remains unknown (Goldenberg et al., 1996; Pastore et al., 1999; Royce et al., 1999; Ness et al., 2003; Allsworth and Peipert, 2007). Studies show African women to be either less likely to harbor H₂O₂-producing lactobacilli which are known to suppress growth of anaerobic bacteria (Antonio et al., 1999) or to have lower concentrations of the same (Anukam et al., 2006; Zhou et al., 2007; McClelland et al., 2009). African women also tend to have significantly higher vaginal pH than do Caucasian women (Ravel et al., 2010). In addition, the prevalence of BV has been shown to be

higher in HIV-infected women compared to HIV-uninfected women (Sewankambo et al., 1997; Jamieson et al., 2001; Warren et al., 2001). From the foregoing information, it is apparent that more studies are needed to complete our understanding of BV in affected sub-populations. Further, it is not known how vaginal microflora varies during the phases of the menstrual cycle.

The goal of the present study was to investigate the composition of vaginal microflora of non-pregnant premenopausal HIV⁺ and HIV⁻ women in a sub-urban population of Kenya by PCR-based identification of culture isolates obtained during the follicular and ovulation phases of the normal menstrual cycle.

MATERIALS AND METHODS

Study population

This was a cross-sectional study nested in an on-going Tigon dysplasia study (TDS) based at Tigon Hospital, Kiambu West district of Kenya. Recruitment of participants took place at the hospital between August 2006 and March 2008. Of the 215 women screened, 100 of them met the inclusion criteria, and only 74 returned for the scheduled six follow-up visits. Specimens were collected at three points of two consecutive menstrual cycles—follicular (day 5-8), ovulation (day 12-15) and luteal (day 19-22) phases. At each phase, the sampling period was spread out within four days to create flexibility for return visits. The participants were asked to mark all the days of menstrual bleeding as a guide to establishing the regularity of the cycle. Following verbal explanation of the study objectives, written consent to participate was obtained for all participants and hard copies of the study explanation issued to them. The documents were available in both English and the most widely used local language, Kiswahili. The study was approved by the ethical review board of Kenyatta National Hospital/University of Nairobi (Registration number P122/8/2005).

During the study, all STIs diagnosed were treated according to the Kenya Ministry of Health national guidelines (2007) for both participants and those excluded from the study. All HIV⁺ women received treatment free of charge, courtesy of the sponsored partnership of Kenya Ministry of Health, University of Nairobi Institute of Tropical and Infectious Diseases and Pathfinder International. At the time of the study, all HIV⁺ persons with a CD₄ count above 200 cells/mm³ were exempted from antiretroviral therapy (ART) according to WHO guidelines (1997). Qualifying premenopausal women, aged between 18 and 45 years were screened for eligibility using a standard face-to-face questionnaire interview. The HIV⁺ women who had CD₄ counts above 250 cells/mm³ and not undergoing ART were recruited. All the participants had regular menses and a documented normal papanicolaou (pap) smear test result performed within one year.

The exclusion criteria were presence of STIs, ongoing pregnancy, use of contraception except condom, previous hysterectomy, delivery or abortion in the preceding three months and use of systemic antibiotics (except cotrimoxazole prophylaxis for HIV⁺ women given under Kenya national guidelines for HIV management). The HIV test was performed in order to group patients as positive and negative.

Following the face-to-face questionnaire interview, subjects provided blood specimens for HIV testing, syphilis and CD₄ count. Vaginal specimens were taken to test for *T. vaginalis*, *C. trachomatis* and *N. gonorrhoeae*. HIV pre- and post-test counselling was offered by a trained nurse.

Screening tests

Study participants were screened for HIV-1 infection using enzyme-linked immunosorbent assay (ELISA) Detect-HIV (BioChem ImmunoSystems, Allentown, PA). Positive samples were confirmed by a second ELISA (Vironostika, BioMérieux, Marnes-la-Coquette, France) (WHO guidelines). Urine pregnancy test was performed using a rapid β -hCG test kit (Plasmatec Laboratory products, Cambridge, UK) and syphilis seroreactivity assessed by the Rapid Plasma Reagin (Becton Dickinson, Baltimore, MD). Confirmatory *Treponema pallidum* haemagglutination assay was not necessary as no seroreactive cases were detected. Two high vaginal swabs were taken for *T. vaginalis* culture (In-Pouch, Biomed Diagnostics, San Jose, CA) and for diagnosis of candidiasis assessed by microscopic examination for presence of budding yeasts or pseudohyphae in a drop of 10% KOH. One endocervical swab was required for combined *C. trachomatis* and *N. gonorrhoeae* polymerase chain reaction (PCR) (Cobas Amplicor, Roche Diagnostics, Basel, Switzerland). The CD₄ count was performed by flow cytometry using specific antibodies (Becton Dickinson), and stained samples analysed using a FacsCaliber instrument and the CELLQuest Software (Becton Dickinson).

Follow-up sample collection

The 74 women (41 HIV⁺ and 33 HIV⁻) followed up had two high vaginal swabs taken at subsequent visits as follows: with a non-lubricated speculum in place, sterile cotton swabs were consecutively inserted into the vaginal vault. Each swab was rotated against the lateral vaginal wall at the mid-portion of the vault and carefully removed to prevent contamination with the vulva and introitus microflora. The first swab was used to make a Gram stain. The second swab was carried to the laboratory in Amies transport medium (Nuova Aptaca, Canelli, Italy) for anaerobic culture which was performed at follicular (day 5-8) and ovulation (day 12-15) phases. Culture swabs were processed in the microbiology laboratory within 4 h of collection. Gram stain specimens were analysed for the composition of the vaginal microflora by microscopy according to the Nugent criteria (Nugent et al., 1991). An additional category of the gram stains known as grade 0 was included to represent the smears lacking bacteria cells.

Culture and identification of isolates

The swabs were streaked onto Columbia agar (Becton Dickinson) supplemented with 5% sheep blood (obtained freshly defibrinated from the Kabete Veterinary farm, University of Nairobi, Kenya) and incubated anaerobically (GasPak EZ Anaerobe Container System) at 37°C. After 4 days of incubation, all isolates with different colony morphology were selected for species identification. DNA was extracted by simple alkaline lysis as follows: each colony selected for its unique morphology was suspended in 20 μ l sodium dodecyl sulfate (SDS) lysis buffer (0.25% sodium dodecyl sulphate in 0.05 N NaOH), heated at 95°C for 15 min and diluted with 180 μ l double distilled water. The t-DNA-PCR and capillary electrophoresis were carried out as described previously (Baele et al., 2000; Baele et al., 2002). Species identification of culture isolates was achieved by comparing their respective t-DNA-PCR fingerprints with an existing t-DNA-PCR database using an in-house software program (Baele et al., 2002). The t-DNA-PCR fingerprint database and the software are available upon request to Laboratory for Bacteriology Research, Ghent University, Belgium, where the PCR work was carried out. Isolates that could not be identified in the existing t-DNA-PCR database were sequenced as earlier described (Verhelst et al., 2004).

Statistical analysis

Prevalence rates were compared between groups through Chi-square test or Fischer's Exact Test. Statistical significance was accepted at the significance level $\alpha=0.05$. All analyses were performed with statistical software package PASW v18.0 (Chicago, IL).

RESULTS

Cohort characteristics

Baseline demographic characteristics of 41 HIV⁺ and 33 HIV⁻ women are presented in Table 1. All participants were literate and able to follow the appointment schedule. At enrolment, no significant difference existed in most parameters measured in the two groups. The HIV⁺ women had lower CD₄ counts ($p<0.001$) and condom use was more common among them (61%) compared to the HIV⁻ women (18.2%) ($p=0.0002$). Intake of antibiotic prophylaxis was only in the HIV⁺ group at 78% ($p<0.001$). The Kenya national guidelines for treatment and care for HIV persons allows continuous antibiotic intake for prevention of malaria and recurrent bacterial infections. Of the HIV⁺ participants, 63.4% were married compared to 39.4% HIV⁻ ones ($p=0.001$). The HIV⁺ women had lower levels of schooling ($p=0.005$).

Vaginal microflora and HIV status

Bacterial vaginosis was diagnosed in 17.5% enrolment visits. Table 2 shows the fluctuation in Gram stain score over two menstrual cycles. Respectively 42.4% of HIV⁻ and 31.7% of HIV⁺ women harboured a normal vaginal microflora on all six visits. As shown in Table 3, the number of vaginal swabs with grades I, II and III was similar throughout the phases of the menstrual cycle in both study groups.

Presence of bacterial species according to HIV status

Species cultured and identified in the HIV⁺ and HIV⁻ women are presented in Table 4 and Figure 1. In total, 1020 isolates were cultured. Of these, 26% of the isolates remained unidentified either because no corresponding t-DNA-PCR fingerprint was found in the existing database or no amplification obtained. In total, 51 species were identified, nine of which belonged to the genus *Lactobacillus*, that is, *L. coleohominis*, *L. crispatus*, *L. gasseri*, *L. iners*, *L. jensenii*, *L. mucosae*, *L. reuteri*, *L. salivarius* and *L. vaginalis*. In descending order, the most common lactobacilli were *L. crispatus*, *L. iners*, *L. jensenii* and *L. vaginalis*. Respectively, 54.5% of HIV⁻ and 60.9% of HIV⁺ women were colonized by lactobacilli at least one visit, and 9.1 and 29.3% at least three visits. *Lactobacillus crispatus* was cultured on three out of four

Table 1. Descriptive characteristics of the study population at enrolment, expressed as percentages.

Parameter	Category	HIV ⁺ (41)	HIV ⁻ (33)	p value
Age (years)	21-28	14.6	24.2	0.4
	29-36	53.7	39.4	
	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	
Systolic Blood Pressure (mm Hg)	90-110	34.1	30.3	0.8
	111-130	51.2	48.5	
	131-150	12.2	15.2	
	151-170	2.4	3.0	
	> 170	0.0	3.0	
Diastolic Blood Pressure (mm Hg)	60-69	12.2	15.2	0.8
	70-79	43.9	36.4	
	80-89	29.3	27.3	
	90-99	14.6	18.2	
	> 100	0.0	3.0	
Pulse (beats/min)	< 60	0.0	3.0	0.3
	60-69	9.8	24.2	
	70-79	39.0	30.3	
	80-89	36.6	33.3	
	90-99	14.6	6.10	
	> 100	0	3.0	
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	

Table 1. Contd.

Parameter	Category	HIV ⁺ (41)	HIV ⁻ (33)	p value
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	

Table 2. Percentages of the fluctuation of the Gram stain scores of HIV⁻ and HIV⁺ women taken at six visits each, spanning two menstrual cycles.

Parameter	HIV ⁻		HIV ⁺	
	No (27)	Yes (6)	No (17)	Yes (24)
Condom use (total number)	No (27)	Yes (6)	No (17)	Yes (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 3. Distribution of Nugent scored vaginal microflora grades among HIV⁺ and HIV⁻ women during different phases of two menstrual cycles. Percentages in parentheses.

Grade	HIV ⁺ (n = 41)			HIV ⁻ (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

Table 4. Percentage of 250 visits during which 44 HIV⁺ and 33 HIV⁻ women with a positive culture.

Species	HIV ⁺ women ^a	HIV ⁻ 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 microaerophilus</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 asaccharolyticus</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

^a142 visits; ^b108 visits.

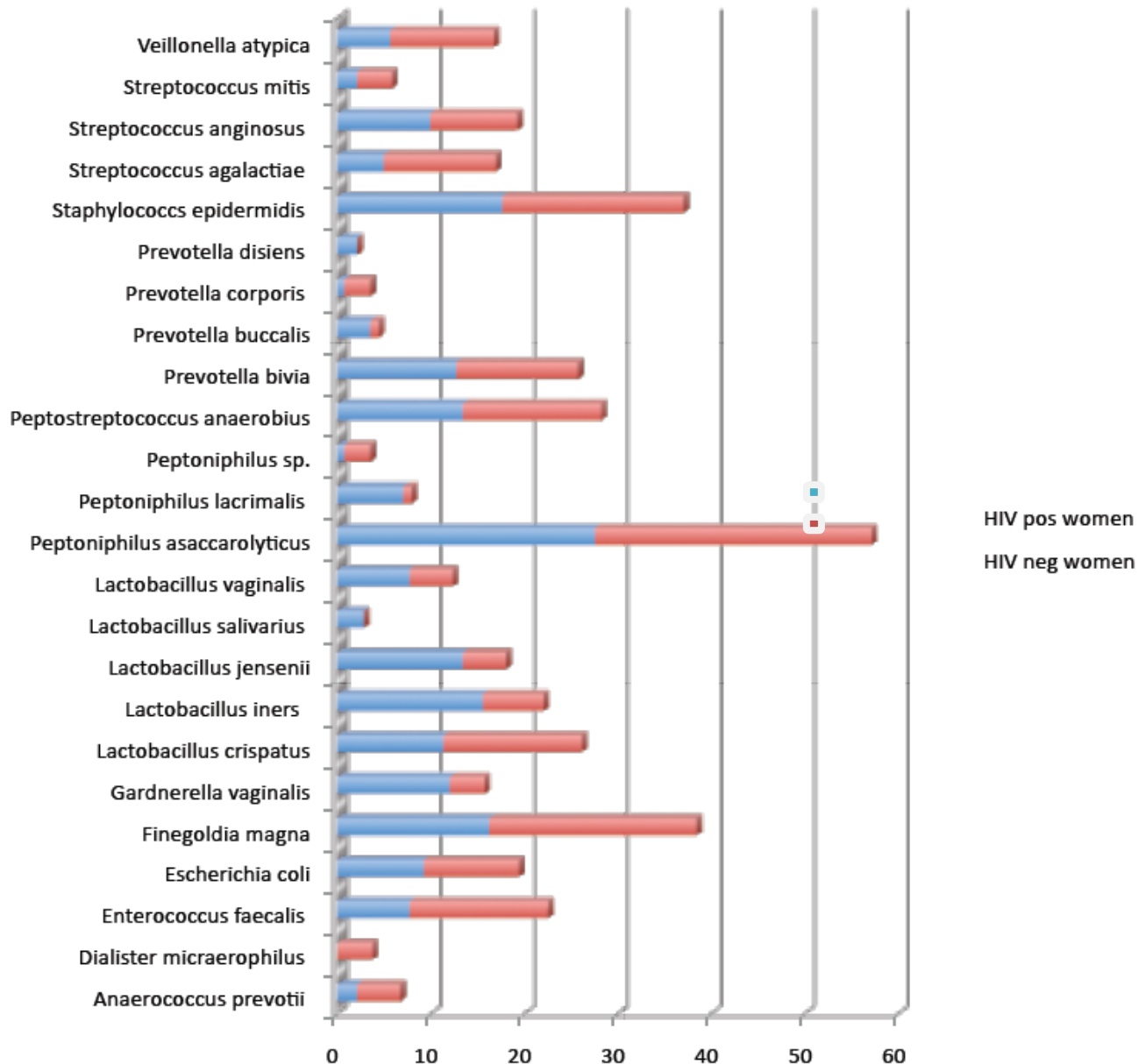


Figure 1. Bacterial species present in more than 2% of the 74 women studied.

visits for two women and it occurred twice in five women and once in 11 women. *Lactobacillus iners* was cultured on three visits for two women, 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 one visit in four women. Two species occurred more frequently in the HIV+ women compared to HIV- women: *L. iners* (15.5% vs 6.5%; $p=0.02$) and *L. jensenii* (13.4% vs 4.6%; $p=0.01$). *G. vaginalis* and *P. lacrimalis* occurred more frequently in HIV+ women (respectively 12% vs 3.7% and 7% vs 0.9%; $p=0.01$). *D. micraerophilus* was found more frequently in the HIV- group (3.7% vs 0%; $p=0.02$) as was *S.*

agalactiae (12% vs 4.9%; $p=0.04$).

DISCUSSION

The objective of this study was to compare vaginal microflora of HIV+ and HIV- Kenyan women of reproductive age by means of culture and Nugent scoring of Gram stains. There were existing differences in the species present in the two groups of women. This confirms findings of Spear et al. (2008) who identified a trend towards increased diversity in HIV+ women, suggesting that HIV infection may be associated with

changes in the diversity of genital microflora.

Presence of BV has been associated with increased susceptibility to acquisition of STIs (Martin et al., 1999; Sewankambo et al., 1997; Kaul et al., 2007). Since BV increases the risk of HIV acquisition, it would be expected that higher rates of BV be confirmed among HIV⁺ women. However, in this study, no significant difference was found in the rates of BV in HIV⁺ and HIV⁻ women. This agrees with findings of Demba et al. (2005) and Watts et al. (2006), but is in contradiction to observation of other researchers (Jamieson et al., 2001; Sewankambo et al., 1997; Warren et al., 2001) who found prevalence of BV to be higher among HIV-infected women compared to HIV-uninfected women.

Possible explanation for the lack of correlation between the prevalence of BV and HIV status observed in the current study may be due to differences in antibiotic intake between HIV⁺ and HIV⁻ women. The imbalance of vaginal microflora in this HIV⁺ group may further be explained by the fact that 78% of them were on cotrimoxazole (trimethoprim-sulfamethoxazole) prophylaxis. Although this antibiotic has been shown to reduce mortality of HIV patients and to have a beneficial effect by decreasing the rate of CD₄ cell depletion (Mermin et al., 2004), use of antibiotics decimates H₂O₂-producing lactobacilli in the vagina (Vallor et al., 2001; Hummelen et al., 2010). Co-trimoxazole is the prophylaxis antibiotic approved by the Kenya Ministry of Health (2007) and is readily available to HIV⁺ persons in health centers. It is used similarly in other sub-Saharan African countries (Hamer and Gill, 2008). Since the HIV⁺ women continue to have more lactobacilli despite continuous intake of cotrimoxazole, it is suggested that resistance to this antibiotic may have been attained. A previous study found that women with BV have decreased colonization rates for H₂O₂-producing strains of lactobacilli and increased colonization rates for non-H₂O₂-producing strains (Onderdonk et al., 2003; Ferris et al., 2007). This observation is supported by the present findings that *L. iners*, *G. vaginalis* and *P. lacrimalis* occurred more frequently in HIV⁺ women regularly using antibiotics.

Some studies found infection with HIV-1 to be associated with abnormal vaginal microflora. Sewankambo et al. (1997) who studied 4718 women in a community survey in rural Uganda, concluded that loss of lactobacilli or presence of BV may increase susceptibility to acquisition of HIV-1. Jamieson et al. (2001) and Warren et al. (2001) while studying different aspects of a comparable high-risk cohort of 1288 women in the USA, found HIV infection to be positively correlated with presence of BV. However, they could not determine whether HIV-infected women have a higher incidence of BV or more persistent infections. Participants of the said study were not excluded on account of low CD₄ counts or other STIs like in the current one. Jamieson et al. (2001) found that immunocompromised women (CD₄ cell count < 200 cells/μL) were more likely to have prevalent and persistent BV than HIV-infected women with higher CD₄

cell counts (>500 cells/μL), but not more likely to have incident infections. This could be explained by the fact that the immunocompromised women were probably on HAART and the accompanying cotrimoxazole prophylaxis (Hamer and Gill, 2008; Watts et al., 2006). In the current cohort, none of the 74 women were immunocompromised or on ART, as illustrated by the fact that, of the 41 HIV⁺ women, 56.1% had CD₄ cell count in the range 250-500 cells/μL and the rest in the range 501-1500 cells/μL.

Further to the differences in immune status and therapeutic intervention between the two study groups, differences in sexual dynamics may also play a role. At the time of the study, most of the HIV-infected women had one (75.6%) or no (24.4%) sexual partners while 63.4% were married. This may partly explain why no differences were detected in the prevalence of BV between the HIV- infected and uninfected subjects. Confounders not measured but varying between populations may also contribute to the difference in findings. In the current study more HIV⁺ women were married, which may imply that marriage increased the risk of exposure to HIV infection in this population. This finding is consistent with data obtained by the Kenya Aids Indicator Survey (KAIS, 2007). Compared to the HIV⁻ group, the HIV⁺ women had lower levels of schooling, which may contribute to the observed lower rate of formal employment of HIV⁺ women (24.4% compared to 42.4% HIV⁻ women). A previous study in Kenya (Steele et al., 2004) found genital hygiene practices to be associated with resource access factors such as higher education and income. Further, a more recent study clearly shows low socio-economic status to be associated with prevalence of BV in African American women (Allsworth and Peipert, 2007). The presence of BV is believed to predispose women to acquisition of HIV-1 (Sewankambo et al., 1997), whereas the psychosocial stress created by being HIV infected could predispose to acquisition of BV (Nansel et al., 2005), thus creating a complex inter-relationship between the two infections.

In agreement with other culture-based studies of African populations (Martin et al., 1999; McClelland et al., 2009), the concentrations of vaginal *Lactobacillus* species were comparatively low, and prevalence of abnormal vaginal microflora and BV-associated species high in the current study group. High rates of BV were previously observed in cohorts of African women (Sewankambo et al., 1997; Taha et al., 1998) and in Kenya (Bukusi et al., 2006; McClelland et al., 2009). It appears, therefore, that African women have lower concentration of lactobacilli in their vaginal microflora, which may explain the high rates of BV in this population. Reasons for this are unclear, although vaginal hygiene practices and sexual behaviour may play a role. No significant effect of the menstrual cycle on the prevalence of BV was observed. Earlier studies found quantities of lactobacilli to decrease during menstruation (Keane et al., 1997) and vaginal microflora to be more unstable during the first week (Morison et al., 2005). The current study

found almost equal numbers of grade I to III samples evenly spread out in the three sampling points at follicular (day 5-8), ovulation (day 12-15) and luteal (day 19-22) phases. This difference in observations may be explained by the varying time frame of sample collection in the current study. Keane et al. (1997) had a daily collection of the vaginal swabs while Morison et al. (2005) collected a sample on alternate days. As recent report by Brotman et al. (2010) showed that episodes of BV may be missed if specimens are collected at weekly or monthly intervals since fluctuations occur more frequently, and in some episodes remission may occur without intervention. However the current study had the strength of having accounted for most known behavioural confounders commonly associated with increased dynamism of the vaginal microflora (such as age, douching, contraceptive use, vaginal lubricants, irregular condom use, STIs and multiple partners) (Schwebke et al., 1999). This makes the observation of almost equal numbers of grade I to III samples evenly spread out in the three sampling points of the menstrual cycle more reliable.

Demba et al. (2005), also using culture methods, found *Prevotella* and *Bacteroides* species to occur more frequently in Nigerian HIV⁺ women. The current study found these species to be equally distributed among both HIV⁺ and HIV⁻ groups. Other bacteria isolated in the vaginas of the participants that are not associated with normal vaginal microflora include the streptococci. *S. agalactiae* was most common in the HIV⁻ group. Most of the other species identified occurred at more or less the same frequency in the two groups, and in other cases where a species was altogether absent in one group, statistical significance in the differences was not attained.

The finding that the most abundant *Lactobacillus* species in the grade I category were *L. iners* (17.1%), *L. crispatus* (16.4%), *L. jensenii* (12.5%) and *L. vaginalis* (9.2%), in that order, are largely in agreement with previous studies of African women. In Nigeria, using denaturing gradient gel electrophoresis, one group (Anukam et al., 2006) found *L. iners* and *L. gasseri* to be the predominant species while in Uganda others (Jin et al., 2007) found *L. reuteri*, *L. crispatus*, *L. vaginalis* and *L. jensenii* to be most abundant using culture and PCR identification as in the current study. A more recent study conducted in Kenya (Matu et al., 2009) found *L. jensenii* to be the most abundant species using culture and phenotypic identification methods. In Belgian women, *L. iners* is more dominant in altered vaginal microflora (De Backer et al., 2007). One group (Anukam et al., 2005; Anukam et al., 2006) did not however detect lactobacilli in BV cases whereas in the current study, of the nine *Lactobacillus* species detected, eight of them were present in the non-grade I samples. The causes of racial difference in concentrations of lactobacilli remain unknown. A highly intriguing observation came from a study of Nigerian women (Anukam et al., 2006) that found only 3% of the healthy vaginal microflora to be dominated by *L. crispatus*, while *L. iners* was present in

64%. Overall African women tend to have lower concentrations of lactobacilli (McClelland et al., 2009). This observation may be a possible explanation for the high prevalence of BV among African women (Allsworth and Peipert, 2007; Bukusi et al., 2006) since it is known that most *L. iners* strains are very weak H₂O₂ producers. In contrast, *L. crispatus* is known to produce large amounts of H₂O₂ and as such provides better colonization resistance (Antonio et al., 1999; Vallor et al., 2001; Verstraelen et al., 2009; Verstraelen et al., 2010).

Low concentrations of lactobacilli in Kenyan women have been observed by other researchers (McClelland et al., 2009). Further, among 185 and 241 Nigerian women respectively, two studies found 12 and 10 *Lactobacillus* spp. to be present (Anukam et al., 2005; Anukam et al., 2006). An earlier study comparing Korean and Ugandan women found that vaginal microflora may vary in women of different geographical communities (Jin et al., 2007). In a US study African American women with BV were found to be predominantly colonized by *Mobiluncus* spp., based on gram staining (Royce et al., 1999). Researchers in Nigeria (Anukam et al., 2006) and The Gambia (Demba et al., 2005) found *Mycoplasma hominis* to be commonly associated with BV. In the present study *Mobiluncus* spp. was cultured in only one subject in the non-grade I category despite the observation of *Mobiluncus*-like organisms in the vaginal Gram-stained smears. *M. hominis* was not cultured at all. Although this could point to culture bias, the Demba group (Demba et al., 2005) also failed to isolate *Mobiluncus* in the Nigerian population of 277 women using a 72 h incubation protocol. Like their group, the current study used Columbia blood agar and longer incubations of 96 h.

Some species identified in greater proportions in either the HIV⁺ or HIV⁻ women did not attain statistical significance due to small sample size. Studies of this nature conducted on a larger sample size will help to draw conclusions on other types of microflora predominant in HIV⁺ and HIV⁻ women, thus enabling more specific approaches to probiotic therapy. Probiotic therapy may need to be geographically determined in order to take care of differences in the species and populations of *Lactobacillus*. Treating BV in HIV⁺ women would subsequently reduce the risk of mother-to-child-transmission.

Conclusions

The current data provide evidence that differences exist in the species that colonize the vaginas of HIV⁺ and HIV⁻ women in the course of the normal menstrual cycle. BV was equally high in HIV⁺ and HIV⁻ women. *Lactobacillus* and BV-associated species were cultured more frequently in HIV⁺ women. Predominant *Lactobacillus* in culture were *L. crispatus*, *L. iners*, *L. jensenii* and *L. vaginalis*. These Kenyan women had lower concentrations of lactobacilli in vaginal microflora than observed

in previous studies of Caucasian women.

ACKNOWLEDGEMENTS

Teresa N. Kiama is grateful to the inter-University collaboration between the University of Nairobi and the Belgium government (VLIR) for the doctoral scholarship. Additional funding was provided by Bijzonder Onderzoeksfonds (BOF08/GOA/002) of Ghent University, Belgium. The authors wish to thank all the women participants for their time and effort in the study; the members of Tigoni district hospital staff and the VLIR Reproductive Health project.

REFERENCES

- Allsworth JE, Peipert JF (2007). Prevalence of bacterial vaginosis: 2001-2004 National Health and Nutrition Examination Survey data. *Obstet. Gynecol.* 109:114-120.
- Antonio MA, Hawes SE, Hillier SL (1999). The identification of vaginal *Lactobacillus* species and the demographic and microbiologic characteristics of women colonized by these species. *J. Infect. Dis.* 180:1950-1956.
- Anukam K, Osazuwa E, Ahonkhai I, Reid G (2005). 16S rRNA gene sequence and phylogenetic tree of *Lactobacillus* species from the vagina of healthy Nigerian women. *African Journal of Biotechnology Vol 4*:1222-1227.
- Anukam KC, Osazuwa EO, Ahonkhai I, Reid G (2006). *Lactobacillus* vaginal microbiota of women attending a reproductive health care service in Benin city, Nigeria. *Sex Transm. Dis.* 33:59-62.
- Atashili J, Poole C, Ndumbe PM, Adimora AA, Smith JS (2008). Bacterial vaginosis and HIV acquisition: a meta-analysis of published studies. *Aids* 22:1493-1501.
- Baele M, Vanechoutte M, Verhelst R, Vancanneyt M, Devriese LA, Haesebrouck F (2002). Identification of *Lactobacillus* species using tDNA-PCR. *J. Microbiol. Methods.* 50:263-271.
- Baele M, Vanrobaeys M, Vanechoutte M, De Herdt P, Devriese LA, Haesebrouck F (2000). Genomic fingerprinting of pigeon *Streptococcus gallolyticus* strains of different virulence by randomly amplified polymorphic DNA (RAPD) analysis. *Vet. Microbiol.* 71:103-111.
- Bolton M, van der Straten A, Cohen CR (2008). Probiotics: potential to prevent HIV and sexually transmitted infections in women. *Sex. Transm. Dis.* 35:214-225.
- Brotman RM, Ravel J, Cone RA, Zenilman JM (2010). Rapid fluctuation of the vaginal microbiota measured by Gram stain analysis. *Sex Transm. Infect.* 86:297-302.
- Bukusi EA, Cohen CR, Meier AS, Waiyaki PG, Nguti R, Njeri JN, Holmes KK (2006). Bacterial vaginosis: risk factors among Kenyan women and their male partners. *Sex Transm. Dis.* 33:361-367.
- Cherpes TL, Melan MA, Kant JA, Cosentino LA, Meyn LA, Hillier SL (2005). Genital tract shedding of herpes simplex virus type 2 in women: effects of hormonal contraception, bacterial vaginosis, and vaginal group B *Streptococcus* colonization. *Clin Infect Dis.* 40:1422-1428.
- De Backer E, Verhelst R, Verstraelen H, Alqumber MA, Burton JP, Tagg JR, Temmerman M, Vanechoutte M (2007). Quantitative determination by real-time PCR of four vaginal *Lactobacillus* species, *Gardnerella vaginalis* and *Atopobium vaginae* indicates an inverse relationship between *L. gasseri* and *L. iners*. *BMC Microbiol.* 7:115.
- Demba E, Morison L, van der Loeff MS, Awasana AA, Gooding E, Bailey R, Mayaud P, West B (2005). Bacterial vaginosis, vaginal flora patterns and vaginal hygiene practices in patients presenting with vaginal discharge syndrome in The Gambia, West Africa. *BMC Infect. Dis.* 5:12.
- Farquhar C, Mbori-Ngacha D, Overbaugh J, Wamalwa D, Harris J, Bosire R, John-Stewart G (2010). Illness during pregnancy and bacterial vaginosis are associated with in-utero HIV-1 transmission. *Aids* 24:153-155.
- Ferris MJ, Norori J, Zozaya-Hinchliffe M, Martin DH (2007). Cultivation-independent analysis of changes in bacterial vaginosis flora following metronidazole treatment. *J. Clin. Microbiol.* 45:1016-1018.
- Goldenberg RL, Klebanoff MA, Nugent R, Krohn MA, Hillier S, Andrews WW (1996). Bacterial colonization of the vagina during pregnancy in four ethnic groups. *Vaginal Infections and Prematurity Study Group. Am. J. Obstet. Gynecol.* 174:1618-16121.
- Hamer DH, Gill CJ (2008). Balancing individual benefit against public health risk: the impact of cotrimoxazole prophylaxis in HIV-infected patients on antimicrobial resistance. *Am. J. Trop. Med. Hyg.* 79:299-300.
- Hummelen R, Fernandes AD, Macklaim JM, Dickson RJ, Chantalucha J, Gloor GB, Reid G (2010). Deep sequencing of the vaginal microbiota of women with HIV. *PLoS One.* 12:5(8):e12078.
- Jamieson DJ, Duerr A, Klein RS, Paramsothy P, Brown W, Cu-Uvin S, Rompalo A, Sobel J (2001). Longitudinal analysis of bacterial vaginosis: findings from the HIV epidemiology research study. *Obstet. Gynecol.* 98:656-663.
- Jin L, Tao L, Pavlova SI, So JS, Kiwanuka N, Namukwaya Z, Saberhein BA, Wawer M (2007). Species diversity and relative abundance of vaginal lactic acid bacteria from women in Uganda and Korea. *J. Appl. Microbiol.* 102:1107-1115.
- K, Temmerman M, Ronald AR, Moses S (2007). Prevalent Herpes simplex virus type 2 infection is associated with altered vaginal flora and an increased susceptibility to multiple sexually transmitted infections. *J. Infect. Dis.* 196:1692-1697.
- KAIS (2007). Kenya AIDS Indicator Survey.
- Kalra A, Palcu CT, Sobel JD, Akins RA (2007). Bacterial vaginosis: culture- and PCR-based characterizations of a complex polymicrobial disease's pathobiology. *Curr. Infect. Dis. Rep.* Kaul R, Nagelkerke NJ, Kimani J, Ngugi E, Bwayo JJ, Macdonald KS, Rebbapragada A, Fonck
- KDHS, (2007) Ministry of Planning and National Development. Kenya Demographic and Health Survey. Marrazzo JM (2004). Evolving issues in understanding and treating bacterial vaginosis. *Expert. Rev. Anti. Infect. Ther.* 2:913-22.
- Keane FE, Ison CA, Taylor-Robinson D (1997). A longitudinal study of the vaginal flora over a menstrual cycle. *Int. J. STD. AIDS.* 8: 489-94.
- Martin HL, Richardson BA, Nyange PM, Lavreys L, Hillier SL, Chohan B, Mandaliya K, Ndinya-Achola JO, Bwayo J, Kreiss J (1999). Vaginal lactobacilli, microbial flora, and risk of human immunodeficiency virus type 1 and sexually transmitted disease acquisition. *J. Infect. Dis.* 180:1863-8.
- Matu MN, Orinda GO, Njagi EN, Cohen CR, Bukusi EA (2009). In vitro inhibitory activity of human vaginal lactobacilli against pathogenic bacteria associated with bacterial vaginosis in Kenyan women. *Anaerobe.* 16: 210-5.
- McClelland RS, Richardson BA, Hassan WM, Graham SM, Kiarie J, Baeten JM, Mandaliya K, Jaoko W, Ndinya-Achola JO, Holmes KK (2009). Prospective study of vaginal bacterial flora and other risk factors for vulvovaginal candidiasis. *J. Infect. Dis.* 199:1883-1890.
- Mermin J, Lule J, Ekwaru JP, Malamba S, Downing R, Ransom R, Kaharuza F, Culver D, Kizito F, Bunnell R, Kigozi A, Nakanjako D, Wafula W, Quick R (2004). Effect of co-trimoxazole prophylaxis on morbidity, mortality, CD4-cell count, and viral load in HIV infection in rural Uganda. *Lancet* 364:1428-1434.
- Morison L, Ekpo G, West B, Demba E, Mayaud P, Coleman R, Bailey R, Walraven G (2005). Bacterial vaginosis in relation to menstrual cycle, menstrual protection method, and sexual intercourse in rural Gambian women. *Sex. Transm. Infect.* 81:242-7.
- Nansel TR, Doyle F, Frederick MM, Zhang J (2005). Quality of life in women undergoing medical treatment for early pregnancy failure. *J Obstet Gynecol Neonatal Nurs.* 34:473-81.
- Ness RB, Hillier S, Richter HE, Soper DE, Stamm C, Bass DC, Sweet RL, Rice P (2003). Can known risk factors explain racial differences in the occurrence of bacterial vaginosis? *J. Natl. Med. Assoc.* 95: 201-12.
- Nugent RP, Krohn MA, Hillier SL (1991). Reliability of diagnosing bacterial vaginosis is improved by a standardized method of Gram stain interpretation. *J. Clin. Microbiol.* 29:297-301. Onderdonk AB,

- Lee ML, Lieberman E, Delaney ML, Tuomala RE (2003.) Quantitative microbiologic models for preterm delivery. *J. Clin. Microbiol.* 41: 1073-1039.
- Pastore LM, Royce RA, Jackson TP, Thorp JM Jr, Savitz DA, Kreaden US (1999). Association between bacterial vaginosis and fetal fibronectin at 24-29 weeks' gestation. *Obstet. Gynecol.* 93: 117-23.
- Paxton LA, Sewankambo N, Gray R, Serwadda D, McNairn D, Li C, Wawer MJ (1998). Asymptomatic non-ulcerative genital tract infections in a rural Ugandan population. *Sex Transm. Infect.* 74: 421-425.
- Ravel J, Gajer P, Abdo Z, Schneider MG, Koenig SSK, McCulle SL, Karlebach S, Gorle R, Russell J, Tacket CO, Brotman RM, Davis CC, Ault K, Peralta L, Forney LJ (2010). Vaginal microbiome of reproductive-age women. *Proceedings of the National Academy of Sciences*, Direct Submission.
- Royce RA, Thorp J, Granados JL, Savitz DA (1999). Bacterial vaginosis associated with HIV infection in pregnant women from North Carolina. *J. Acquir. Immune. Defic. Syndr. Hum. Retrovirol.* 20: 382-6.
- Schwebke JR (2005). Abnormal vaginal flora as a biological risk factor for acquisition of HIV infection and sexually transmitted diseases. *J. Infect. Dis.* 192: 1315-7.
- Schwebke JR, Richey CM, Weiss HL (1999). Correlation of Behaviour with microbiological changes in vaginal flora. *J. Infect. Dis.* 180: 1632-6.
- Sewankambo N, Gray RH, Wawer MJ, Paxton L, McNaim D, Wabwire-Mangen F, Serwadda D, Li C, Kiwanuka N, Hillier SL, Rabe L, Gaydos CA, Quinn TC, Konde-Lule J (1997). HIV-1 infection associated with abnormal vaginal flora morphology and bacterial vaginosis. *Lancet* 350:546-50.
- Sha BE, Zariffard MR, Wang QJ, Chen HY, Bremer J, Cohen MH, Spear GT (2005). Female genital-tract HIV load correlates inversely with *Lactobacillus* species but positively with bacterial vaginosis and *Mycoplasma hominis*. *J. Infect. Dis.* 191:25-32.
- Shin LY, Kaul R (2008). Stay it with flora: maintaining vaginal health as a possible avenue for prevention of human immunodeficiency virus acquisition. *J. Infect. Dis.* 197:1355-7.
- Spear GT, Sikaroodi M, Zariffard MR, Landay AL, French AL, Gillevet PM (2008). Comparison of the diversity of the vaginal microbiota in HIV-infected and HIV-uninfected women with or without bacterial vaginosis. *J. Infect. Dis.* 198:1131-1140.
- Spear GT, St. John E, Zariffard MR (2007). Bacterial vaginosis and human immunodeficiency virus infection. *AIDS Res. Ther.* 4:25.
- Srinivasan S, Liu C, Mitchell CM, Fiedler TL, Thomas KK, Agnew KJ, Marrazzo JM, Fredricks DN (2010). Temporal variability of human vaginal bacteria and relationship with bacterial vaginosis. *PLoS One.* 5:e10197.
- Steele MS, Bukusi E, Cohen CR, Shell-Duncan BA, Holmes KK (2004). Male genital hygiene beliefs and practices in Nairobi, Kenya. *Sex Transm. Infect.* 80:471-476.
- Taha TE, Hoover DR, Dallabetta GA, Kumwenda NI, Mtimavalye LA, Yang LP, Liomba GN, Broadhead RL, Chipangwi JD, Miotti PG (1998). Bacterial vaginosis and disturbances of vaginal flora: association with increased acquisition of HIV. *Aids* 12:1699-1706.
- Vallor AC, Antonio MA, Hawes SE, Hillier SL (2001). Factors associated with acquisition of, or persistent colonization by, vaginal lactobacilli: role of hydrogen peroxide production. *J. Infect. Dis.* 184:1431-6.
- van de Wijgert JHHM, Mason PR, L. Gwanzura, Mbizvo TM, Chirenje ZM, Iliff V, Shoboski S, Padian NS (2000). Intravaginal practices, vaginal flora disturbances, and acquisition of sexually transmitted diseases in Zimbabwean women. *J. Infect. Dis.* 181:587-594.
- Verhelst R, Verstraelen H, Claeys G, Verschraegen G, Delanghe J, Van Simaey L, De Ganck C, Temmerman M, Vaneechoutte M (2004). Cloning of 16S rRNA genes amplified from normal and disturbed vaginal microflora suggests a strong association between *Atopobium vaginae*, *Gardnerella vaginalis* and bacterial vaginosis. *BMC Microbiol.* 4:16.
- Verhelst R, Verstraelen H, Claeys G, Verschraegen G, Van Simaey L, De Ganck C, De Backer E, Temmerman M, Vaneechoutte M (2005). Comparison between Gram stain and culture for the characterization of vaginal microflora: definition of a distinct grade that resembles grade I microflora and revised categorization of grade I microflora. *BMC Microbiol.* 5:61.
- Verstraelen H, Verhelst R, Claeys G, De Backer E, Temmerman M, Vaneechoutte M (2009). Longitudinal analysis of the vaginal microflora in pregnancy suggests that *L. crispatus* promotes the stability of the normal vaginal microflora and that *L. gasseri* and/or *L. iners* are more conducive to the occurrence of abnormal vaginal microflora. *BMC Microbiol.* 9:116.
- Verstraelen H, Verhelst R, Vaneechoutte M, Temmerman M (2010). The epidemiology of bacterial vaginosis in relation to sexual behaviour. *BMC Infect. Dis.* 10:81.
- Warren D, Klein RS, Sobel J, Kieke B Jr, Brown W, Schuman P, Anderson J, Cu-Uvin S, Mayer K, Jamieson DJ, Holmberg S, Duerr A (2001). A multicenter study of bacterial vaginosis in women with or at risk for human immunodeficiency virus infection. *Infect. Dis. Obstet. Gynecol.* 9:133-141.
- Watts DH, Springer G, Minkoff H, Hillier SL, Jacobson L, Moxley M, Justman J, Cejtin H, O'Connell C, Greenblatt RM (2006). The occurrence of vaginal infections among HIV-infected and high-risk HIV-uninfected women: longitudinal findings of the women's interagency HIV study. *J. Acquir. Immune. Defic. Syndr.* 43:161-168.
- WHO, World Health Organization Guidelines for persons living with HIV (1997).
- Wiesenfeld HC, Hillier SL, Krohn MA, Landers DV, Sweet RL (2003). Bacterial vaginosis is a strong predictor of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* infection. *Clin. Infect. Dis.* 36: 663-8
- Zhou X, Brown JC, Abdo Z, Davis CC, Hansmann MA, Joyce P, Foster JA, Forney LJ (2007). Differences in the composition of vaginal microbial communities found in healthy Caucasian and black women. *International Society for Microbial Ecology.* 1:121-133.