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MULTIPLEX PCR TECHNIQUE TO IDENTIFY OF SUDANESE FEMALES VAGINAL MICROBIOTA

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ABSTRACT

Background: Microbiota of healthy women changes from birth to menopause. During their reproductive years, female's normal flora is dominated by *Lactobacillus* species, females whom are identified with vaginal infections are characterized by lacking *Lactobacillus* species and the community is composed of facultative and strictly anaerobic bacteria. Recently, a new level of detailed description of the vaginal microbiome has been attained using quantitative, real-time, PCR targeting key bacterial genomes. In this study we have used 16S RNA sequencing to identify the Sudanese flora within females in their reproductive age. **Objectives:** Molecular characterization of the vaginal microbiome among Sudanese females within the reproductive age and the micro-flora associated with bacterial vaginosis (BV) in Khartoum State, Sudan using molecular diagnosis (conventional PCR technique) of vaginal samples. **Materials and Methods:** This is a descriptive cross sectional and molecular laboratory based study; was carried out in Khartoum state-Sudan selected two major reproductive centres. Vaginal swab samples were collected from participants who visited reproductive centres in period between March 2016 to March 2017. The DNA was then extracted from the swab samples and 16S RNA sequencing was performed. **Results:** Multiplex PCR screened for **100** vaginal swabs samples. *Lactobacillus* species was found to be the most dominant organism in all specimens except of samples with bacterial vaginosis (B.V) which was found to be dominated with the genus *Gardnrella*. Contraceptive and skin smoking (Dukhan) were found to have a significant impact on the presence of vaginal flora

with P-value of 0.000 and 0.003 respectively. **Conclusions and Recommendations:** Sudanese female's flora is mostly dominated by *Lactobacillus* species among normal and asymptomatic females and *Gardnrellla* sp. Among females with B.V. more studies and metagenomics sequencing is recommended for better identification.

KEYWORDS: *microbiota, PCR, Lacbacillus, Bacterial Vaginosis.*

INTRODUCTION

Microbes are recognized as playing an important role in the balance between health and disease in the human host. While the recent advances in DNA sequencing techniques have revealed that an increased diversity in the gut microbiota is generally linked to less susceptibility to disease, the opposite seems to be true for the vaginal microbiota (Petrova et al., 2017).[1] The vagina has a complex ecosystem of microflora which changes throughout life, from birth to menopause (Bitew et al., 2017).[2] This microbiome consists of species and genera which typically do not cause symptoms or infections in women with normal immunity. The vaginal microbiome is dominated by *Lactobacillus* species (Döderlein bacilli) in the normally healthy vagina of women of reproductive age (Petrova et al., 2015).[3] Bacterial vaginosis (BV) is a clinical condition often referred to as an overgrowth of normal bacteria in the vagina. It is the most common vaginal infection among women of reproductive age. These species metabolize glycogen and breaking it down into glucose and lactic acid (Petrova et al., 2015).[3]

In recent years, new techniques are being used to better define the clinical expression known as BV-PCR qualification of *Gardnerella vaginalis*, and Lactobacillus species, microarray analysis and 454-sequencing are being used to understand more about the vaginal microbiome. However, within the Sudan primary healthcare setting, no PCR and sequence techniques are currently available, and therefore understanding the vaginal microbiome is a new challenge. In the human vagina, the resident microbes reside in and on the surface of the vaginal epithelium relying primarily on host products for nutrients and in turn establishing a first line of defense against exogenous disturbances. The broad goal of our study was to characterize the vaginal microbiome under healthy and altered states using high-throughput sequencing techniques.

MATERIALS AND METHODS

Study design, area and population

This is a descriptive cross sectional and molecular laboratory based study; was carried out in Khartoum state-Sudan and other Sudan region selected two major reproductive centres from March 2016 to March 2017. Vaginal swab samples were collected from participants who visited reproductive centres at research time for routine check and/or suspected to have BV. All participant are within reproductive age ranged from >15 up to 50 years. Questionnaire were filled from participants directly after verbal and written consent acceptance was included in research.

Sample collection: 100 Vaginal swabs were collected from females The DNA was extracted using Intron DNA Extraction Kit.

Molecular Identification

Identifection of all collected strain was performed geno-typically by multiplex PCR (Table 1 and 2), reaction conditions were prepared by using ready master mix (APSLABS, India). 0.5µl of each primer (pf/pr1, 2 &3primers pair) and 1 µl of template DNA in a total 25 µl for ITS amplification. PCR performed as following: pre-denaturation at 94C̊ for 10 min, 30 cycles of denaturation at 94 C̊ for 40sec, annealing at 55 C̊ for 30 sec and extension at 72 C̊ for 30sec, with final extension step of 72 C̊ for 5min. The PCR product was analysed with gel electrophoresis.

Table 1: Primers specific to multiplex bacterial vaginosis and V1-V3 region along with sequence and amplicon size (bp).

Organism	Forward and reverse sequence (5`-3`)	Amplicon size (bp)	Reference
Gardnerella vaginalis	F-ACTCCTRCGGGAGGCAGCAG- R-GACGGGCGGTGTGTRCA	338 bp	Oakley B. B. et al., 2008(35)[4]
Atopobium vaginae	F-AGTTTGATCCTGGCTCAG R-ATTACCGCGGCTGCTGG	534 bp	Verhelst R., et al., 2004(93)[5]
UNIVERSAL V1-V3 Streptococcus sp, S.aureus, Lactobacilli sp, Atopobium sp, Sneathia sp, Eggerthella sp, Megasphara sp and Fusobacterium sp	F-ATTACCGCGGCTGCTGG R-CCTACGGGAGGCAGCAG	341-534 bp	Ling Z., 2010(55)[6]

Table 2: Best sequence.

QUGP Primer	primer sequence (5`-3`)
QUGP-Rn1	GGCTACCTTGTTACGACTTC
QUGP-F1	AGTTTGATCCTGGCTCAG
QUGP-Fn3	CAGGATTAGATACCCTGGTAGTCC
QUGP-F4	CCGCCTGGGGAGTACG
QUGP-Fn5	ACTCCTACGGGAGGCAGCAG
QUGP-Fn-6	CCAGCAGCCGCGGTAATAC

RESULTS

Clinical and sociodemographic data characteristics: A total of 100 vaginal smears were collected from Sudanese female mean age (30.7±7.05) years from different health centres in the period from March 2016 to March 2017, Khartoum and \Khartoum suburbs. (Table.3). Eighty four out of hundred (84%) of the females used antimicrobials 3 months before sample collection. Most were taking antimicrobials for UTI treatment and only 10 of them used antimicrobials for BV treatment. Eight women used hormonal contraceptives, 5 women were not sexually active and; 87 women reported using no form of contraceptives. Approximately more than half of women non-pregnant (66/100), and 34 women were pregnant. None of the women in our study reported a history of gonorrhea or syphilis infections; whereas 17 women suffering from irritation or discomfort symptoms, 15 women reported with abnormal discharge and only 6 women abnormal discharge with odour. Circumcision was reported in 83/100 women. With regards to skin smoke (Sudanese tradition/ Dukhan) 87/100 women were applying it regularly (Table 4).

Socio-demographic characteristics

In total, 100 vaginal smears were collected from Sudanese female mean age (30.7±7.05) years from different centres in the period between March (2016 to 2017) from Khartoum suburbs. The marital status, majority of our population was married 97.0% and only 3.0% single. The highest education level attained of our study population was, 10 was alliterated, 19 did not complete high school, 16 completed their secondary education, 39 completed their graduate degree and 16 completed post-graduate studies. (Table 3).

Table (3): Socio-demographic characteristics of female participants at Khartoum city, March 2016- March 2017.

Female characteristics	No. (%) n=100
Age group	
16-26 years	31 (31.0%)
27-36 years	44 (44.0%)
37-46 years	20 (20.0%)
>46 years	5 (5.0%)
Mean ± SD (Range)	30.72± 7.05
Marital status	
Single	3 (3.0%)
Married	97 (97.0%)
Educational level	
Illiterate	10 (10.0%)
Primary	19 (19.0%)
Secondary	16 (16.0%)
University	39 (39.0%)
Post graduate	16 (16.0%)

- 1N indicates number of participants (total 100 women).

Table 4: Clinical and Behavioural characteristics of female participants at Khartoum city, 2016-2017.

Clinical characteristics	No. (%) n=100
Pregnancy	
Pregnant	34 (34.0%)
Non-pregnant	66 (66.0%)
Hormonal contraceptive use	
Yes	8 (8.0%)
No	92 (92.0%)
Antibiotics use (in the past three months)	
Yes	84 (84.0%)
No	16 (16.0%)
Symptoms	
Abnormal discharge	15 (15.0%)
Abnormal discharge with odor	6 (6.0%)
Irritation or discomfort	17 (17.0%)
Behavioral Characteristics	
Circumcision	Yes: 83 (83.0%) No: 17 (17.0%)
Skin smoke	Yes: 87 (87.0%) No: 13 (13.0%)

- 1N indicates number of participants (total 100 women).

Microscopic characteristics

The vaginal swab Gram stained smears were examined microscopy; 62.0% of the women

were positive for single microorganisms, 34.0% mixed microorganisms, 5.0% for yeast cells, with only two patients having uncountable pus cells. 'Clue cells' were seen in 32.0% of the Gram-stained smears. BV prevalence as determined by Nugent's score of 7–10 was 17.0%, 10.0% women had 'intermediate flora' (score 4–6) and 73.0% had 'normal flora' (score 0–3). **(Table 5, Fig: 1)**

Gram positive bacilli (*Lactobacillus* sp.) were present as predominant vaginal microflora which represented 95.0% of all women vaginal smears. Whereas, gram variable bacteria **(***Gardnerella* sp.) were present in 17.0% of women followed by gram positive cocci (*Staphylococcus/Streptococcus*) *and Bacteroides sp/Prevotella sp* in 13.0% and Coliform sp in 12.0%. Additionally; *Peptostreptococcus sp/ Gemella sp, Veillonella sp, Neisseria gonorrhoeae* and *Mobiluncus sp* were represented in 7, 4, 2 and 1 respectively of women with scanty numbers. **(**Table 5**)**

Table (5): Microscopic examinations of Gam stain and Nugent score category of vaginal swab samples, at Khartoum city, 2016-2017.

Microscopic characteristics of	**Total N=100**
Gram reaction	
Pus cell presence	2 (2.0%)
Clue cells presences	32 (32.0%)
Single microorganisms	62 (62.0%)
Mixed microorganisms	34 (34.0%)
Yeast cells presences	5 (5.0%)
Microorganism identified:	
G +ve Bacilli- *Lactobacillus sp*	**95 (95.0%)**
G +ve Cocci-*Staphylococcus/Streptococcus*	13 (13.0%)
G +ve Rods- *Bacteroides sp/Prevotella sp*	13 (13.0%)
G -ve bacilli- Coliform sp.	12 (12.0%)
G +ve Coccobacilli- *Gardnerella sp*	**17 (17.0%)**
G +ve Coccobacilli- *Peptostreptococcus sp/ Gemella sp*	7 (7.0%)
G -ve Cocci- *Veillonella sp*	4 (4.0%)
G -ve Diplococci- *Neisseria gonorrhoeae*	2 (2.0%)
G -ve rods- *Mobiluncus sp*.	1 (1.0%)
Nugent score category:	
Inconsistent for BV (0 – 3)	73 (73.0%)
Intermediate for BV (4 – 6)	10 (10.0%)
Consistent for BV (7 – 10)	17 (17.0%)

- 1N indicates number of participants (total 100 women)

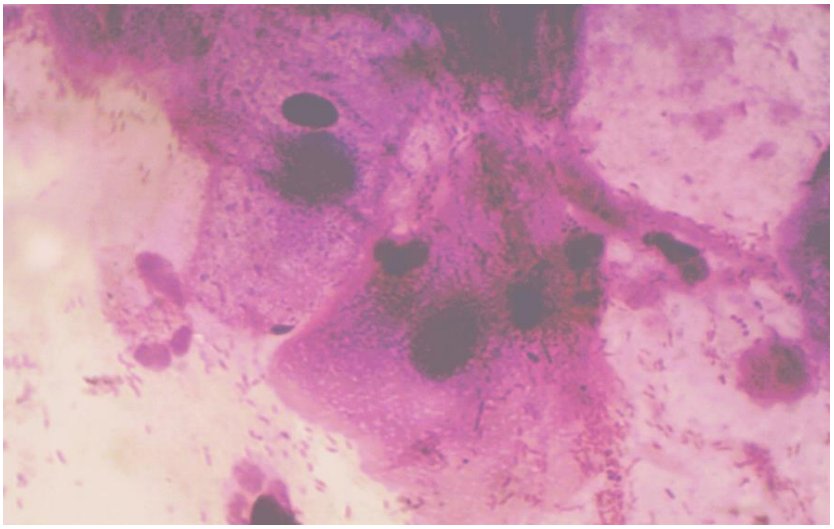

Fig. 1: shows sample number 56 gram stain microscopy.

PCR screening outcome

According the multiplex PCR screening outcome of vaginal microbiota from 100 women during the study, the total genomic DNA were extracted and detect *G. vaginalis, A. vaginae* and V1-V3 region bacteriome. Agarose gel electrophoresis showing result of samples were produced a different band sizes <100bp for V1 region, 341-534bp for V3 region, 338bp for *G.vaginalis* and 534bp for *A.vaginae*. Result of samples 8S, 9S and 17S were produced a different band sizes with the Pf/$Pr_{1,}$ 2&3 primers pair Shows in Fig (2), 17 samples shows bands for both *G.vaginalis* and *A.vaginae* however 100 samples all shows different bands position.

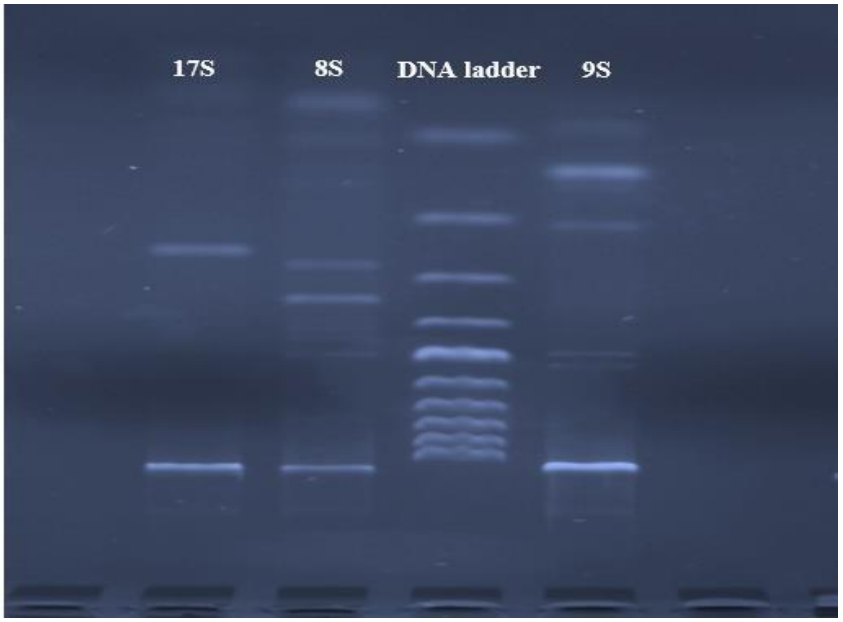

Fig. (4.2): Agarose gel.

Electrophoresis showing result of samples 8S, 9S and 17 S were produced a different band sizes with the Pf/$Pr_{1,2\&3}$ primers pair multiplex PCR detection of B.V and vaginal microbiota.

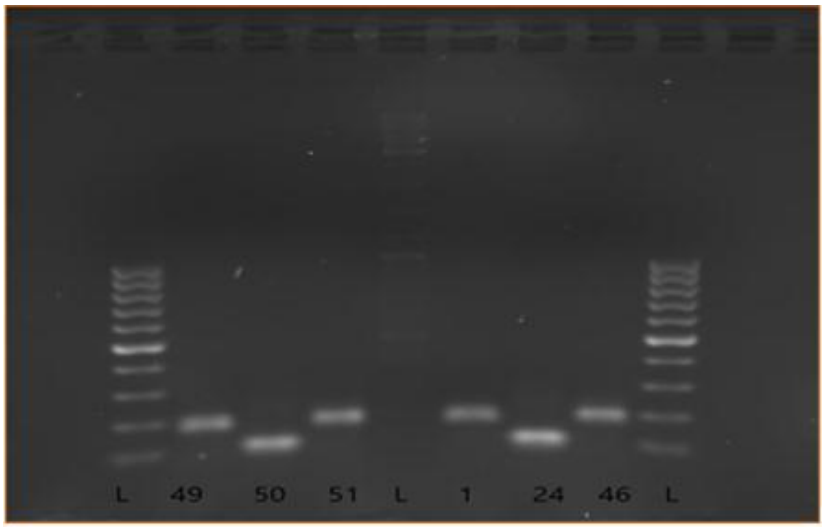

Fig 3: Picture showed samples (1, 46, 49 and 51) with band 181bp (primer F5,R6) referred to *Lactobacillus spp* and samples Number 50 and 24 with band size 107bp (primer F3, R4) referred to *Cocobacillus spp.*

DISCUSSION

In this study, we compared vaginal bacteriome community structures between Sudanese women with and without clinically defined BV and synthesized publicly available data to better understand the diversity of healthy and diseased vaginal bacterial groups.

Lactobacilli play a major role in maintaining the urogenital health by preventing the overgrowth and invasion of pathogenic bacteria by competing with other microorganisms for nutrients.[7,8] *G. vaginalis* was included in this study, because it has been described as the most prevalent pathogenic bacteria in patients diagnosed with BV.[9] Nevertheless, frequent isolation of this species from seemingly healthy women has cast doubt on this claim.

Identification of vaginal bacteriome using gram stain method with Nugent criteria

The Gram stained smears offers an alternative to use of the composite criteria with Nugent *et al method*[10,11], which is widely used particularly for research studies, requires counting of bacteria. Also widely used to diagnose bacterial vaginosis according to the proportions of different cellular morphologies seen in gram-stained smears of vaginal samples. The weighted score computed using these criteria is thought to reflect the relative abundance of the following morph types: Lactobacilli, *G. vaginalis* or *Bacteroides* (small gram-variable

rods or Gram-negative rods), and curved gram-variable rods. Using Nugent's score, a BV was found in 17.0% of women in our study. This compares to the range (20–49%) reported from other African populations.[12,13] There was agreement with studies done in among pregnant women in Nigeria and west Africa[12,14] as BV had been highly prevalent.

The BV is associated with a change in vaginal ecology, resulting in overgrowth of *G. vaginalis, Bacteroides sp/Prevotella sp, Mobiluncus sp, Veillonella sp* and *A. vaginae* as anaerobic bacteria associated with bacterial vaginosis, which replacing or overlapping the lactobacillus dominated flora of the normal vagina. The BV being reported as 10–20% in sexually active women[15] and higher in women attending specialized clinics for sexually transmitted infections (STI) or for termination of pregnancy.[16] As the majority of study group were non-pregnant 66%. The bacteria associated with BV with serious health problems including adverse pregnancy outcomes such as preterm delivery and low birth weight babies[17], as well as an increased risk of pelvic inflammatory disease (PID) and post- abortal sepsis.[18]

In practice and in our experience, 'morphotypes' are often difficult to assign to one of these groups. Also, some genera and species that are clearly associated with bacterial vaginosis, like *Peptostreptococcus* sp and *A. vaginae* are not included in the Nugent score.[19] Furthermore, there was several problems in the interpretation of smears regarding Nugent criteria, the presence of different *Lactobacillus* cell types in smears from patients with bacterial vaginosis can lead to assignation to grade II, whereas patients without bacterial vaginosis but with smears with more than 300–500 pleomorphic *Lactobacillus* cells may be regarded as containing *G. vaginalis*, also because some of these cells are very small.[20] Additionally, the Nugent scoring system conflates women with potentially very different vaginal microflora in a single category.

A variety of those tests, which reflect the changes in vaginal ecology, have been used to diagnose BV. Whereas identification and suspected of the bacteriome from Gram stain appearance was a poor specific. Relying on gram reaction on bacterial morphology as most of anaerobic bacteria of the vagina gram positive rod/coccobacilli and for *lactobacillus* sp gram positive bacilli as it was difficult to identify without culture isolation as complementary to our result we perfumed through Molecular diagnostic (PCR technique) for V1 and V3 region to more specifying the bacteria phylum, genus and species level.

Investigate Sudanese vaginal microbiome associated with BV among the study group

One non-pregnant woman's Gram stain and Nugent profile mostly consisted of uncountable pus cell without microorganisms and clue cell detected group II. This women had metagenomic profiles dominated by *Lactobacillus* sp, *S. anginosus* with other anaerobic cocci. Whereas *S. anginosus* is a pathogen implicated in urogenital and gastroinstestinal tract infections. Although *S. anginosus* has been found in the human vaginal and urinary microbiome[21], in our study a genome of an *S. anginosus* and *Veillonellaceae* was sequenced with pus cell there is strong association found either due circumcision complication leading to UTI[22,23] or fecal contamination of the vagina.[24] Researches relating to relationship of *Veillonellaceae* due to fecal bacterial transfers to genital microflora responsible for non-specific vaginosis.

Bacterial vaginosis (BV) is a poly-microbial syndrome characterized by a shift in vaginal flora from a predominant population of lactobacilli to their gradual or total replacement with anaerobes such as *Gardnerella vaginalis, Prevotella, Bacteroides* and *Mobiluncus* species (spp), and with other bacteria including *Mycoplasma* and *Ureaplasma* species.[25] BV is one of the most frequent conditions encountered in sexually transmitted diseases (STD), genitourinary medicine (GUM) or other reproductive health clinics throughout the world. BV appears to be particularly common in sub-Saharan Africa where several studies have reported high prevalence rates, ranging from 20–49% among women presenting to STD clinics with vaginal discharge[12,26], from 21–52% among pregnant women attending antenatal clinics[14,27], and from 37–51% in community-based studies.[28] The Nugent's method has been extensively validated in 3rd countries where numerous vaginal flora studies have been conducted, but little is known of the pattern of vaginal micro-flora associated with BV in Sudan. The characterization of vaginal micro-flora is an important step in understanding the pattern of flora associated with BV. This study; may help investigate the significance of this condition in clinical pathology and for targeting treatment, while behavioral factors such as circumcision and skin smoke have been suggested as important factors that might influence vaginal flora composition, as there is no data is available from Sudanese populations.

We also saw an association between our BV-associated Nugent score, and *L. iners, L. vaginalis,* G. vaginalis, *Gemella* sp and Prevotella sp. These results support the pathogenetic role these bacterial species play in BV with/without symptoms. This is further portrayed by the association seen between numbers of *G. vaginalis* related episodes in the past year. This

data to date gives us a much broader, more nuanced understanding of the organism clusters seen in women with/ without BV.

Past literature has associated *Lactobacillus* species with health, and their predominance in the vaginal environment is thought to promote a healthy microbiota However, *L. iners* has been seen at high levels in both women with and without BV. As indicated earlier, *L. iners* is suggested to be a transitional bacterial species, pushing a healthy microbiota into an unhealthy state.[29,30,31] BV has been noted to be complex and diverse in the literature, and we see our findings are in line with previous research as these bacterial species have been suggested to be associated with BV.[32,33,34] Ravel et al. noted a relationship between high Nugent scores and *Aerococcus*, *Gardnerella*, *Gemella*, *Mobiluncus*, *Prevotellaceae* and *Veillonellaceae* which is similar to our findings.[35]

The most prevalent phylotypes in group III include *Gardnerellaceae*, *Prevotella*ceae *and Gemellaceae*. In contrast, the most prevalent bacterial clones in Subject 11h and 21s include ***L. iners*** *and L. vaginalis* with *Gemellaceae* noticed in both women, thereby illustrating the differences in bacterial phylotypes between two subjects with symptomatic BV. The bacterial diversity associated with a symptomatic BV in this study is substantially lower than the diversity detected in symptomatic BV, was found to be *A.vaginae* with lower *lactobacillus* sp, and *G.vaginalis* percentages. The different results observed in this study could also be due heterogeneity in vaginal microbiota among women. Between different clones were sequenced from five women resulting in 9 to 12 bacterial phylotypes per subject. Two women had vaginal bacterial biotas dominated by *L. iners*, *Gardnerellaceae, A.vaginae*, *Prevotellaceae* and *Veillonellaceae in a symptomatic BV,* while three novel taxa were associated with the symptomatic BV of women vagina including large number of *Lactobacillaceae* and *Gardnerellaceae*. However, these bacteria have been associated with BV by other investigators.[36-39] As standard objective criteria were not used for the diagnosis of BV, it is difficult to draw conclusions from this study about the constituents of the normal vaginal bacterial biota. Whereas in this small sample set, the dominant vaginal bacteriome varied from women to women, also with notable variance in *Lactobacillus* sp. However, these investigations did not include parallel sampling of both non-pregnant and pregnant subjects, nor from multiple behavioral factors.

Our study found that where Gram-staining led to a classification of 'intermediate flora' by Nugent's score, this was reflected in the microbiological findings, which were 'intermediate'

quantitatively and qualitatively between 'normal' and 'BV' categories and distinct from them. This supports the validity of the classification and could indicate that the 'intermediate' flora precedes the development or follow the resolution of frank BV. Three of the 11 women had intermediate BV Nugent score and a combination of eleven phylum is detect as the majority *Lactobacillaceae* and other bacteria including *Gardnerella sp, Gemellaceae Ureaplasma sp* and *A.vaginae*. *A. vaginae* and *G. vaginalis* have been shown to be present in 78% to 96% of BV samples, in contrast to 5% to 10% of normal flora samples.[37,40] Conversely, Menard et al. detected *A. vaginae* in 69% of samples from women without BV, suggesting that the mere detection of *A. vaginae* has a poor predictive value for BV.[12] Nevertheless their results showed that quantification of *A. vaginae* bacteria is a good predictor, since higher levels were detected in BVpositive samples.[37]

Hillier et al. reported that the prevalence of *U. urealyticum* was 78% in pregnant women with normal flora and was significantly higher, at 92%, in pregnant women with BV.[41,42] Results reported by R Capoccia et al. were similar in that women with BV had a higher *U. urealyticum* carriage rate, at 65%, than did normal women, who had a carriage rate of 48%; however, the difference was not significant.[43]

These studies found a strong association between BV and the isolation of *G vaginalis*, anaerobic gram-negative rods belonging to the genera *Prevotellaceae, Veillonellaceae, Ureaplasma urealyticum*, and often *Mobiluncus* spp. A lower concentration of facultative species of *Lactobacillus* among women with BV in comparison to women with a normal flora was noted in this study. *Lactobacilli* are reported to play an important role in the maintenance of normal vaginal flora.[44] Furthermore, differences in the vaginal microbial composition between ethnic groups may potentiate their predisposition to bacterial vaginosis and infections during pregnancy due to differences in community resilience, which describes the ability of a given community to resist stresses and perturbations and return to a stable equilibrium.[45]

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