Original Article

Molecular Identification of Sudanese Females' Vaginal Microbiota and Microbiota associated with Bacterial Vaginosis, Khartoum, Sudan, 2018

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Abstract

Background: The microbiota of healthy women changes from birth to menopause. During their reproductive years, females' normal flora is dominated by Lactobacillus species. Females who are identified with vaginal infections are characterized by lacking Lactobacillus species and the vagina would be dominated by facultative and strictly anaerobic bacteria. Recently, a new detailed description of the vaginal microbiome has been attained using quantitative, real-time Polymerase Chain Reaction (PCR) targeting key bacterial genomes. In this study, we have used 16S Ribonucleic acid (RNA) sequencing to identify the Sudanese flora and flora associated with bacterial vaginosis within females in their reproductive age.

Materials and Methods: This is a descriptive cross-sectional and molecular laboratory-based study; carried out in one reproductive health Centre and two health care facilities in Khartoum State, Sudan. Hundred vaginal swab samples were collected from the participants who visited the Centres. Out of the 100 serial vaginal swabs, 11 samples were randomly selected. The DNA was then extracted from the swab samples and 16S RNA sequencing was performed. The samples were classified into four groups.

Results: *Lactobacillus* species was found to be the most dominant organism in all four groups except for the group with bacterial vaginosis (BV) which was found to be dominated by the genus *Gardnrella* and *Lactobacillus inners*. Factors such as usage of contraceptives and some Sudanese traditions like circumcision and skin smoking (Dukhan) have significant effect on the flora (p-value 0.045and 0.03 respectively)

Conclusions: Sudanese female flora is dominated mainly by *Lactobacillus* species among normal and asymptomatic females, while *Gardnrellla* sp. was dominant among females with BV. This is consistent with the global picture despite the differences in customs and traditions of Sudanese women, which was expected to affect the types of vaginal bacteria. Further studies and metagenomic sequencing are needed for better identification.

Key words: *Bacterial Vaginosis, Microbiota, 16S RNA, Lacbacillus*

Introduction:

The vagina has a complex ecosystem of microflora that changes throughout life, from birth to menopause (1). 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 (2). 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 break it down into glucose and lactic acid (2).

With the development of high throughput sequencing (HTS), 454 pyrosequencing (3), and Illuminasequencing (4), the tools available for studying the human microbiota, have taken a new major step forward.

For the analysis of bacterial communities, HTS is used in two separate ways; 16S rRNA sequencing uses PCR amplification, of one or more variable regions, in order to identify the microbiota present and their relative abundances. The advantage of sequencing the amplified 16S rRNA is the depth of the investigation, that even bacteria representing less than 0.01% of the bacterial abundance can be detected with a reasonable number of sequences from each sample at a low cost per sample (Fredricks, 2011(5).

In Sudan there is a huge variation among different tribes in their customs and diets, and hence different biota is expected to present. In this study we aim to isolate and identify the vaginal bacterial flora of Sudanese women and the flora associated with BV.

Materials and Methods:

Study design, area, and population:

This is a descriptive cross-sectional molecular laboratory-based study carried out in Khartoum State,Sudan. Women attending one major reproductive center (Sudan Fertility Center) and two health centers (Aylafon Health Center and Eid Babiker Health Center) were considered for this research. A self-administered questionnaire was filled by the participants.Different variables such as Skin-smoking (*Dukhan*), cirumcusion, contraceptives use and anti-biotic use, were included to determine the effect on the vaginal flora.Vaginal swab samples were collected from participants.

Sample Size: A hundred consecutive participants who fulfilled the inclusion criteria were included in the study. The vaginal swabs were collected from the mid-vagina using sterile swabs. All samples were preserved at -20˚C and saved until processed. DNA was extracted using the Intron DNA extract Kit. All samples were tested for bacterial genomic presence using the PCR technique. The samples were grouped into according to their Nugent Score ranking (6): (Clinically and microscopically identified as normal, Symptomatic vaginosis, Asymptomatic vaginosis). Eleven samples were selected for sequencing from the identified groups using simple random selection. A simple random selection for the sample

was made after the groups were, identified according to the mircoscopic identification.

Molecular Identification: For identification and phylogenetic classification of pathogens in diverse microbial populations, prokaryotic 16S rDNA was used. The protocol targets the variable V3 and V2 regions of the 16S rDNA for sequencing. Amplicon primers for amplifying a single amplicon of approximately ~460 bp were ordered from (Integrated DNA Technologies, USA). Amplicon library preparation and sequencing were performed at the National Institute for Communicable Disease Sequencing Core Facility in South Africa. (Table 1). Illumina adapter overhang nucleotide sequences were added to the gene-specific primers for compatibility with Illumina index and sequencing adapters. During library preparation using Nextera XT Library Preparation Kit (Illumina, San Diego, CA, USA), the V3 and V2 region of the 16S rRNA is amplified using a limited cycle PCR, followed by the addition of Illumina sequencing adapters and dual-index barcodes to the amplicon target.

Table (1): Amplicon Library Preparation and Sequencing performed at the National Institute for Communicable Disease Sequencing Core Facility, South Africa.

16S Amplicon PCR Forward Primer	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG
16S Amplicon PCR Reverse Primer	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC

16S data analysis:

Following the sequencing, the resulting paired-end reads were checked for quality, trimmed, and analyzed using the CLC Genomics Workbench version 11 (CLC, Bio-QIAGEN, Aarhus, Denmark). The tools from Amplicon-Based Analysis at the CLC Microbial Genomics Module are designed to cluster all reads within a certain percentage of similarity into Operational Taxonomic Units (OTUs) where they are then represented by a single sequence.

The tools clustered reads at some level of similarity into pseudo-species or OTUs, where all reads within for example 97% similarity are clustered together and represented by a single sequence.
Data was analyzed using Statistical Package for Social Sciences (SPSS) version 20. A chi-square test was conducted to establish the relation between the variables.

Results:

Clinical and socio-demographic data: a total of 100 vaginal smears were collected from Sudanese females' mean age (30.7±7.05) from Khartoum and Khartoum suburbs. (Table 2) shows the age groups. Seventy five percent of the participants were in the younger age group of 16 – 36 years. The state of conception, contraceptive use, antibiotic use in the preceding 3 months and clinical symptoms are shown in (Table 3)

Table (2): Age groups of participants of the study population, Khartoum, 2018.

Age groups	n=100 No. (%)
16-26 years	31 (31.0%)
27-36 years	44 (44.0%)
37-46 years	20 (20.0%)
>46 years	5 (5.0%)

Table (3): Behavioral Characteristics of the Participants of the study, Khartoum, 2018

Variable	No (%)
Circumcision	
Yes	83 (83.0%)
No	17 (17.0%)
Skin smoke	
Yes	87 (87.0%)
No	13 (13.0%)

Table (4): Clinical Characteristics of the Participants of the study, Khartoum, 2018. n=100

Variable	No. (%)
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%)

Table (5): Vaginal Bacteriome Profiles Data Frequencies of Symptomatic / asymptomatic BV subjects, Khartoum, 2018.

Genus	15s	49h	10 h	11 h	21 s
	Asymptomatic BV		**Symptomatic BV**		
Frequency at genus level according metagenomic reads					
Actinomycetaceae	19	0	0	0	0
Corynebacteriaceae,	0	1	12	12	25
Propionibacteriaceae	11	0	1	3	4
Gardnerella. Sp	**56**	**43**	**139**	**142**	**281**
Prevotellaceae	15	0	1	3	9
Veillonellaceae	13	1	0	2	2
A.vaginae	0	3	0	0	0
Lactobacillaceae,	25	3	91	295	428
L. iners	**53**	**41**	**1**	**636**	**1485**
L. reuteri	1	0	1	15	17
L. vaginalis	0	0	28	8	36
Ureaplasma.sp	5	0	8	2	10
Gemellaceae	0	1	0	2	2

Other bacteria	115	13	63	138	258
Total	313	105	345	1258	2557

Table (6): **The Effect of Different Variables against the Presence or Absence of Different Micro-organisms that might be found in Normal Females, Khartoum, 2018.**

Variables	Percentage % of identified organisms findings					p-value
	G+ve Bacilli-Lactobacillus	G +ve Cocci-Staphylococcus/ Streptococcus	G +ve Rods-Bacteroides /Prevotella	G+ve Coccobacilli-Gardnerella	Coliform	
Contraceptive yes	1	25	0	0	0	0.000
no	99	75	100	100	100	
Skin smoking yes	93	92	67	50	100	0.003
No	7	8	34	50	0	
Circumcision yes	87	75	67	100	100	0.045
No	13	25	33	0	0	
Antibiotic usage	15	20	0	50	0	0.344

yes						
no	85	80	100	50	100	

PCR screening:

A total of 100 genomic DNA was extracted and amplified by 16S rRNA genes to detect the V1-V3 region bacteriome. Agarose gel electrophoresis showed the result of samples producing different band sizes <100bp for the V1 region, 341-534bp for the V3 region, and 338bp. The result of samples 8S, 9S, and 16S produced different band sizes Fig (1). 17 samples show bands for both G.vaginalis and A.vaginae however 100 samples all show different bands position.

16s rRNA sequencing: Eleven samples of mid-vaginal swabs were randomly selected and divided into 4 groups. The most prevalent phylotypes to genus level in 11 Subjects include for the 16S sequencing the results revealed the presence of: *Gardnerella* sp and *Prevotella* sp. *Lactobacillus* sp, *Streptococcus* sp, and *Staphylococcus* sp as the most predominant phylotypes (Fig 2). In contrast, less prevalent genotypes in 11 Subjects were detected including *Deinococcus* sp, *Meiothermus* sp, *Actinomyces* sp, *Arcanobacterium* sp, *Mobiluncus* sp, *Varibaculum* sp, *Corynebacterium* sp, *Probainobacterium* sp and *Provotella* sp.. Normal vaginal biota profiles represented by Sample 9. Analysis showed the family *Lactobacillaceae* with the most dominant percentage was *Lactobacillus* sp by 95% (Fig 3), followed by 2.7% *L*. iners and 0.9% *L*. reuteri. The 9.5% lowest percentages of other bacteria phylum were *Corynebacterium*, *Propionibacteriaceae*, *Staphylococceae*, *Streptococcaceae* and *Mycoplasmataceae*. Moreover; other types of bacteria showed similar abundance profiles in both target sequence libraries. Samples with symptomatic BV were presented by 45.5% of the 11 samples selected. The most identified organisms were 48.4% *L*. iners and 14.4% *Gardenrella* sp, of the total genomic population. Presentation of other genera and species differs between samples such as *L*. vaginalis and *Gemella* sp, *Peptococcus* sp. *Sporosarcinapasteuri*, *Mycobacterium* tuberculosis. bovis, *Lactobacillus* aviaris, *Carnobacteriumgallinarum*, *Vagococcussalmoninarum* and *Halmonas*. Other genera identified such as *Salmonella* Sp. and *Streptococcus* Sp. were reported as well. (Table 5). Contraceptives, skin smoking, circumcision, antibiotic intake and age groups where analyzed against the presence and nascence of Lactobacilli and other organisms where some showed a significant effect on the type or vaginal flora as shown in (Table 6)

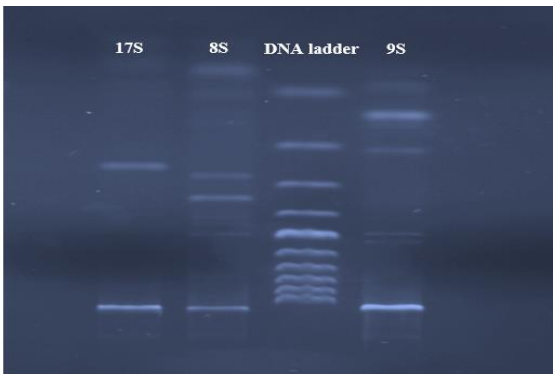

Fig 1: Agarose Gel Electrophoresis showing result of samples 8S, 9S and 17 S, Khartoum, 2018.

As shown in Fig 1, different band sizes were produced with the Pf/Pr$_{1,2\&3}$ primers pair multiplex PCR detection of B.V and vaginal microbial flora.

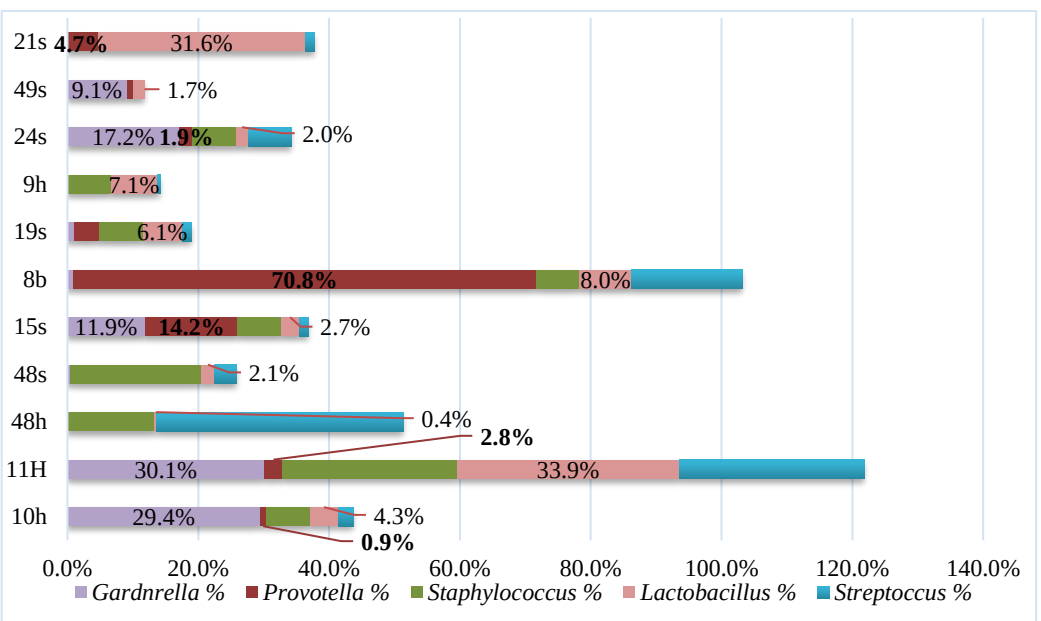

Fig. (2): Vaginal bacteriome profiles t genus level distribution

As shown in fig 2 the microlora of the 11 samples metagenomic reads for V1-V3 hypervariable region for most common genuses read by the 16S sequencing.

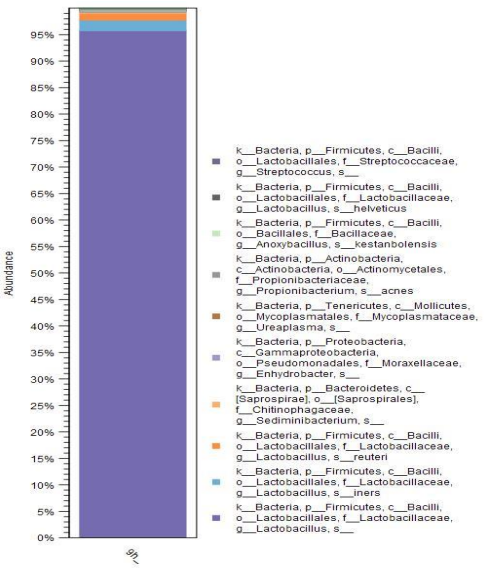

Fig 3: Normal bacterial flora Sample h9

Fig. 3 shows the normal flora and its metagenomic reads for V1-V3 in Sudanese female vaginal flora

Discussion:

According to metagenomic 16s rRNA sequencing; 11 samples of mid-vaginal swabs were randomly selected and grouped into 4 groups as more than 34 phyla of bacteriome were detected in Sudanese women mid-vagina either associated with BV or not. In each sample, Good's coverage was greater than 99% (range: 0.99 - 1.00). The metagenomic 16s rRNA sequencing; was highly robust resulting in a total of 14 phyla in samples 9h and based on a >75% cut-off identity to the V1-V3 gene target identified in our study. Revealing a predominance of 86.8% *Lactobacilli* sp of the normal vaginal biota and 2.7% *L*. iners. *Lactobacilli* sp promote a healthy ecosystem by producing lactic acid, hydrogen peroxide, and bacteriocins that have antimicrobial properties thereby excluding pathogens from this niche. Although both groups of those women were non-pregnant, the behavioral character of the vaginal microbiome gradually revert to baseline characteristics. Several studies have demonstrated that a significant proportion (7–33%) of healthy asymptomatic women lack appreciable numbers of *Lactobacillus* species in the vagina (7, 8), and instead have a vaginal microbiota that consists of other lactic acid-producing bacteria, i.e. species from the genera *Atopobium*, *Leptotrichia* and *Streptococcus* (Aldunate et al., 2015(9). The first report of isolation of *L*. iners in a woman with a normal Nugent score was in 2002 (10) and is one of the organisms most frequently isolated from the vagina of healthy women. (2,11,12,13)

In correlation to circumcision, skin smoke, and pregnancy with vaginal microbiome; 9h women were pregnant, not menopausal at the age of 29 years old, had not had a history of BV, were not circumcised, and were regular skin smoke users. However, microbial communities in the human vagina likely undergo shifts in the representation and abundance of key species over time that are influenced by factors that change vaginal microbiota. Studies of the normal bacterial flora of the female vaginal are primarily limited to the characterization of the types of bacteria present in women who do not have identifiable diseases. Studies by Chaban B *et al.* (14), MacIntyre DA *et al* (15), and Borgogna JL *et al* (16) stated that with the progress of pregnancy, the increased estrogen level has a positive effect on *Lactobacillus* activity and proliferation by increasing glycogen availability. Increased *Lactobacillus* rates are thought to inhibit pathogen growth through the secretion of antibacterial bacteriocins, as well as the production of metabolite, such as lactic acid which helps to maintain a low, hostile pH (17). Our study; showed that the abnormal bacterial colonization decreased significantly (p-value 0.034) (Table 6) as the women uncircumcised.

However, the studies do not address whether some proportion of "healthy" women were in transition to or from BV, or whether they have asymptomatic BV, ie. Abnormal flora but no symptoms due to genetic or other factors.

Our study found that 3/11 women had BV and a combination of eleven phyla is detected as the majority *Lactobacillaceae* and other bacteria including *Gardnerella* sp, *Gemella* sp. *Ureaplasma* sp and *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 (18.19). 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 (20). Nevertheless, their results showed that quantification of *A.* vaginae bacteria is a good predictor since higher levels were detected in BV-positive samples (18).

Hillier et al., 1993, 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 (21.22). 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 (23).

These studies found a strong association between BV and the isolation of *G* vaginalis, anaerobic gram-negative rods belonging to the genera *Prevotellaceae*, *Veillonellaceae*, *Ureaplasmaurealyticum*, and often *Mobiluncus* spp. A lower concentration of facultative species of *Lactobacillus* among women with BV in

comparison to women with normal flora was noted in this study. *Lactobacilli* are reported to play an important role in the maintenance of normal vaginal flora (15). Furthermore, differences in the vaginal microbial composition between ethnic groups may potentiate their predisposition to BV 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 (24).

Conclusion:

Sudanese female flora is dominated mainly by Lactobacillus species among normal and asymptomatic females, while Gardnrellla sp. was dominant among females with BV. This is consistent with the global picture despite the differences in customs and traditions of Sudanese women, which was expected to affect the types of vaginal bacteria. Further studies and metagenomic sequencing are needed for better identification.

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Authors own resources

Conflict of interest

None

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