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WORD COUNT	3400	TIME SUBMITTED	11-NOV-2022 08:53AM
		PAPER ID	92516878

Name of Journal: *World Journal of Clinical Cases*
Manuscript NO: 78920
Manuscript Type: ORIGINAL ARTICLE

Observational Study

Influence of group B *streptococcus* and vaginal cleanliness on the vaginal microbiome of pregnant women

Abstract

BACKGROUND

The vaginal microbiome plays a critical role in the health of pregnant women and their newborns. Group B *Streptococcus* (GBS) and vaginal cleanliness significantly affect the vaginal microecosystem and are closely associated with vaginal diseases.

AIM

To explore the effects of GBS status and vaginal cleanliness on vaginal microecosystems.

METHODS

We collected 160 vaginal swabs from pregnant women and divided them into the following four groups based on GBS status and vaginal cleanliness: GBS-positive + vaginal cleanliness I–II degree, GBS-negative + vaginal cleanliness I–II degree, GBS-positive + vaginal cleanliness III–IV degree, and GBS-negative + vaginal cleanliness III–IV degree. Samples were subjected to 16S rRNA gene amplicon sequencing.

RESULTS

Alpha diversity analysis showed that the Shannon index did not significantly differ between the four groups. We identified significant variation in taxa abundance between the GBS-positive and GBS-negative groups and between the vaginal cleanliness I–II degree and III–IV degree groups. Principal coordinate analysis and non-metric multidimensional scaling analysis further confirmed the microbial diversity of the four groups. Moreover, the linear discriminant analysis demonstrated that *Lactobacillus jensenii* and *Actinobacteria* were strongly associated with GBS-positive status, and *Lactobacillus iners*, *Lactobacillaceae*, *Lactobacillus*, *Lactobacillales*, *Bacilli* and *Firmicutes* were closely correlated with GBS-negative status.

CONCLUSION

GBS status and vaginal cleanliness significantly affect vaginal microbiome differences in pregnant women. Our findings provide instructional information for clinical antibiotic treatment in pregnant women with different GBS statuses and vaginal cleanliness degrees.

INTRODUCTION

The vagina is a complex and sensitive microecosystem controlled by the vaginal anatomy, endocrine regulation, microbial composition, and the local immune system[1]. In dynamic equilibrium, the vaginal microbiome species are mutually interdependent, antagonistic, and controlled by the local immune system, endocrine system, and internal environment[2]. Vaginal pH, estrogen levels, local immunity, *Lactobacillus* species, and vaginal cleanliness play essential roles in maintaining the microecological balance of the vagina[2]. The vaginal microbiome significantly affects vaginal homeostasis. Hence, understanding the vaginal microbiome is essential for vaginal health.

Group B *Streptococcus* (GBS) is a gram-positive bacterium that transiently and asymptomatically colonizes the vagina and gastrointestinal tracts of healthy women.

Thus, it is the principal reason for invasive bacterial disorders in newborns and lethal diseases in infants[3]. Globally, more than three million annual neonatal deaths are caused by GBS infections[4]. The features of GBS have been primarily studied in 4025 women, and a significantly low likelihood of detecting coagulase-negative *Lactobacillus*, *Prevotella*, and *Staphylococcus* has been observed in GBS-positive patients[5,6]. Vaginal cleanliness significantly affects vaginal health. However, the correlation between GBS status and vaginal cleanliness with the vaginal microbiome is still elusive.

In this study, we aimed to investigate the effects of GBS status and vaginal cleanliness on the vaginal microbiome of pregnant women. We successfully identified a novel landscape in which GBS status and vaginal cleanliness significantly affected vaginal microbiome differences in pregnant women.

MATERIALS AND METHODS

Sample collection and study design

A total of 160 vaginal swab samples from pregnant women were collected at our hospital between June 2018 and January 2019. The samples were divided based on GBS status and vaginal cleanliness into the following four groups: GBS-positive + vaginal cleanliness I–II degree (group A, n = 24), GBS-negative + vaginal cleanliness I–II degree (group B, n = 53), GBS-positive + vaginal cleanliness III–IV degree (group C, n = 35), and GBS-negative + vaginal cleanliness III–IV degree (group D, n = 48). Samples were acquired from the patients and healthy participants after obtaining written informed consent. This study was approved by the Ethics Committee of the Shunyi Women and Children's Hospital of Beijing Children's Hospital.

16S rRNA gene amplicon sequencing

DNA samples were obtained from vaginal swabs using a DNA isolation kit (Omega, USA). DNA was quantified using NanoDrop ND-2000 (Thermo Fisher Scientific, USA). The V1–V2 hypervariable regions were also measured. Two standard bacterial 16S rRNA amplicon polymerase chain reaction (PCR) primers were used. A QIAquick PCR

Purification Kit (Qiagen, USA) was used to purify the amplicons, followed by quantification using NanoDrop ND-2000 (Thermo Fisher Scientific, USA). Further, 16S rRNA sequencing was performed using HiSeq 2500 (Illumina, USA).

16S rRNA gene amplicon processing

The 16S rRNA sequencing datasets were filtered and merged using the FLASH method [7]. Sequencing was performed using Quantitative Insights into Microbial Ecology (QIIME, version 1.9.1) software (http://qiime.org/)[8]. Chimeric sequences were deleted applying usearch61 using *de novo* methods[9]. Sequencing was clustered on the 2013 Greengenes (13_8 release) ribosomal database 97% reference dataset (http://greengenes.secondgenome.com/?prefix=downloads/greengenes_database/). Taxonomy was assigned to all operational taxonomic units (OTUs) using the RDP classifier within the QIIME and Greengenes reference datasets[10].

Statistical analyses

Data are presented as mean ± standard deviation. The Wilcoxon rank-sum test was used to evaluate alpha diversity. Analysis of similarities (ANOSIM) of beta diversity matrices was performed to analyze significant differences in microbial communities using QIIME. The microbial biomarkers were analyzed using linear discriminant analysis effect size with the web-based Galaxy interface (http://huttenhower.sph.harvard.edu/galaxy)[11]. The threshold value of > 3 was applied to analyze the discriminative characteristics for the linear discriminant analysis (LDA) score.

RESULTS

Alpha diversity analysis

The effect of GBS status and vaginal cleanliness on the vaginal microbiome was determined using 16S rRNA gene amplicon sequencing. Significantly, the rarefaction curve of the observed species revealed that the depth of 16S rRNA gene amplicon

sequencing satisfactorily demonstrated sequencing diversity among the four groups (Figure 1A). In addition, alpha diversity analysis revealed that the Shannon index failed to exhibit significant differences among the four groups (Figure 1B, Wilcoxon rank-sum test, $P > 0.05$).

Comparison of relative taxa abundance

We then compared the taxa abundance and identified the top ten significant taxa at the phylum, genus, and species levels. At the phylum level, the abundance of *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, *Fusobacteria*, *Tenericutes*, *Proteobacteria*, *Acidobacteria*, *Planctomycetes*, Chloroflexi, and some unidentified bacteria differed between the GBS-positive and GBS-negative groups, as well as between the vaginal cleanliness I–II and III–IV degree groups (Figure 2A). At the genus level, the abundance of *Lactobacillus*, *Gardnerella*, *Bifidobacterium*, *Megasphaera*, *Prevotella*, *Atopobium*, *Aerococcus*, *Sneathia*, *Ureaplasma*, and *Dialister* were different between the GBS-positive and GBS-negative groups and between the vaginal cleanliness degrees I–II and III–IV (Figure 2B). At the species level, the abundance of *Lactobacillus iners*, *Lactobacillus jensenii*, *Prevotella amnii*, *Lactobacillus delbrueckii*, *Atopobium vaginae*, *Prevotella timonensis*, *Aerococcus christensenii*, *Lactobacillus mucosae*, *Lactobacillus reuteri*, and *Sneathia amnii* were different between the GBS-positive and GBS-negative groups and between the vaginal cleanliness I–II and III–IV degree groups (Figure 2C).

Principal coordinates analysis and non-metric multidimensional scaling analysis

We further explored the microbiome differences between the GBS-positive and GBS-negative groups and between the vaginal cleanliness degrees groups I–II and III–IV using beta diversity analyses, including principal coordinates analysis (PCoA) and non-metric multidimensional scaling (NMDS). The PCoA (Figure 3A, unweighted UniFrac distance, ANOSIM) and NMDS analysis showed significant differences between groups A and B, C and D, A and C, and B and D.

Specific taxa analysis

Additionally, we investigated the association of specific taxa with GBS status and vaginal cleanliness based on LDA. Significantly, we observed several specific taxa between the GBS-positive and GBS-negative groups based on groups A and B and groups C and D (Figure 4). *Lactobacillus jensenii* and *Actinobacteria* were closely correlated with GBS-positive status. *Lactobacillus iners*, *Lactobacillaceae*, *Lactobacillus*, *Lactobacillales*, *Bacilli*, *Firmicutes*, and *Bacteria* were strongly associated with GBS-negative status.

DISCUSSION

Diseases associated with vaginal infections cause significant burden to society[12]. Clinical data plus microbiome may be a good model to predict the personalized health status[13]. The vaginal microbiome plays an essential role in women's reproductive health, and GBS status and vaginal cleanliness are crucial in maintaining the vaginal microenvironment[14,15]. However, the impact of GBS status and vaginal cleanliness on the vaginal microbiome remains unclear. In this study, we demonstrated the influence of GBS status and vaginal cleanliness on the vaginal microbiome of 160 vaginal swab samples using 16S rRNA gene amplicon sequencing.

Several 16S rRNA gene amplicon sequencing studies have characterized the vaginal microbiome, including the relationship between the vaginal microbiome and polycystic ovary syndrome[16]. Two 16S rRNA gene investigations have also reported the role of the vaginal microbiome in reproductive-aged women and preterm newborns[17,18]. Another 16S rRNA gene amplicon sequencing identified the vagina-uterine microbiome features and showed that elucidating the vaginal microbiome may help detect prevalent diseases in the upper reproductive tract[19]. Although both GBS and vaginal cleanliness are critical factors for the vaginal microbiome of pregnant women, the combined analysis of GBS and vaginal cleanliness using 16S rRNA gene amplicon sequencing remains limited. Here, we focused on the correlation of GBS status and vaginal cleanliness with the vaginal microbiome. Our data showed no alpha diversity between

the GBS-positive and GBS-negative groups and between the vaginal cleanliness I–II degree and III–IV degree groups. However, the PCoA and NMDS analysis showed significant microbiome differences between the GBS-positive and GBS-negative groups and between the vaginal cleanliness I–II degree and III–IV degree groups, respectively. These data suggest that GBS status and vaginal cleanliness degree can affect the vaginal microbiome, providing new evidence of the association between GBS status and vaginal cleanliness in the vaginal microenvironment. Moreover, our study provides an example of a comprehensive combined analysis of the GBS status and vaginal cleanliness in pregnant women.

Numerous studies have shown that the dominance of a single OTU mainly characterizes the normal vaginal microbiome, most closely related to *Lactobacillus* species[20-22]. The *Lactobacillus* species repress pathogenic microorganisms by maintaining an acidic vaginal pH[23,24]. *Lactobacillus* dominates the healthiest vaginal microbiota, and *Prevotella*, generally identified in the vagina, is associated with bacterial vaginosis and has been correlated with GBS-positive status[25-28]. *Megasphaera* is also associated with a GBS-positive status, which is closely related to bacterial vaginosis[25-27,29]. Nevertheless, investigation of the correlation between vaginal cleanliness and the vaginal microbiome is remarkably limited. In this study, we identified that *Lactobacillus iners*, *Lactobacillus jensenii*, *Prevotella amnii*, *Lactobacillus delbrueckii*, *Atopobium vaginae*, *Prevotella timonensis*, *Aerococcus christensenii*, *Lactobacillus mucosae*, *Lactobacillus reuteri*, and *Sneathia amnii* differed between the GBS-positive and GBS-negative groups, and between the vaginal cleanliness I–II and III–IV degree groups at the species level. Moreover, we found that *Lactobacillus jensenii* and *Actinobacteria* were strongly correlated with GBS-positive status, and *Lactobacillus iners*, *Lactobacillaceae*, *Lactobacillus*, *Lactobacillales*, *Bacilli*, *Firmicutes*, and *Bacteria* were strongly associated with the GBS-negative status. In Pace *et al* study, they assessed positive GBS clinical cultivation, and found a limited number of differentially abundant taxa, including an increased enrichment of *Ureaplasma urealyticum, Corynebacterium glucuronolyticum, Propionibacterium acnes, and Haemophilus haemolyticus*[30]. These data indicated a

correlation between GBS status and vaginal cleanliness in the vaginal microenvironment. Importantly, we present a landscape of the specific vaginal microbiome, such as *Lactobacillus iners*, *Prevotella timonensis*, and *Sneathia amnii*, associated with the GBS status and vaginal cleanliness, and demonstrate the precise vaginal microbiome associated with GBS-positive or -negative status, providing instructional information for clinical antibiotic treatment of pregnant women with different GBS status and vaginal cleanliness degrees.

CONCLUSION

In summary, we discovered that GBS status and vaginal cleanliness significantly affect vaginal microbiota differences in pregnant women. We identified several specific vaginal microbiomes, including *Lactobacillus iners*, *Prevotella timonensis*, and *Sneathia amnii*, in patients with varying GBS statuses. We also found that *Lactobacillus jensenii* and *Actinobacteria* were particularly associated with GBS-positive status, and *Lactobacillus iners*, *Lactobacillaceae*, *Lactobacillus*, *Lactobacillales*, *Bacilli*, *Firmicutes*, and *Bacteria* strongly correlated with GBS-negative status. Our findings provide new insights into understanding the vaginal microenvironment, presenting a landscape of the association of GBS status and vaginal cleanliness with the vaginal microbiome of pregnant women. Our results provide instructional information for clinical antibiotic treatment in pregnant women with different GBS statuses and vaginal cleanliness degrees.

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Figure Legends

Figure 1 Alpha diversity analysis. A: Rarefaction curves of observed species of the four groups in the 16S rRNA gene amplicon sequencing are shown; B: The Shannon index was assessed using alpha diversity analysis of the four groups in the 16S rRNA gene amplicon sequencing, Wilcoxon rank-sum test, $P > 0.05$.

Figure 2 Comparison of relative taxa abundance. A-C: The taxa abundance was compared among the four groups at the phylum (A), genus (B), and species (C) levels.

Figure 3 Principal coordinates analysis and non-metric multidimensional scaling analysis. A: The Principal coordinates analysis based on the unweighted UniFrac distance is shown. Red, blue, green, and orange represent groups A, B, C, and D, respectively. The distance of the points represents comparability; B: The non-metric multidimensional scaling analysis based on the microbial clusters is shown. Red, blue, green, and orange represent groups A, B, C, and D, respectively. The distance of the points represents comparability;

Figure 4 Specific taxa analysis. A-D: The correlation of specific taxa with GBS status was analyzed using linear discriminant analysis (LDA). The LDA biomarkers and trees are shown.

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