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Original Article

Associations between bacterial vaginosis, candida vaginitis, trichomonas vaginalis, and vaginal pathogenic community in Chinese women

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Abstract: Background: To investigate the associations between Vaginal Pathogenic Community with Bacterial vaginosis, Candida vaginitis, and Trichomonas vaginalis in Chinese women. Method: In this experiment, ten BV, nine VVC, eight TV patients, and four non-infected healthy women were recruited. The vaginal samples were collected from the vaginal orifice, the middle of the vagina, and vaginal fornix from every participant and conducted with next-generation sequencing (NGS). The NGS was based upon the analysis of bacterial 16S rRNA genes by using the Illumina Miseq system. Results: No significant difference in microbiome community structures was observed for the three sampling sites from the same subject. Compared with the healthy population, patients with BV and TV showed more diverse symptoms and had a lower amount of *Lactobacillus* but a higher number of BV-related bacteria like *Atopobium*, *Dialister*, *Sneathia*, *Mobiluncus*, and *Prevotella*. On the contrary, the species composition of the VVC group is relatively simple, which has a significantly high abundance of *Lactobacillus.* Eight genera, including *Arcanobacterium*, *Clostridium*, *Moryella*, *Mobiluncus, Shuttleworthia*, *Dialister*, *Bulleidia,* and *Megasphaera,* were closely correlated with BV. Among vaginal pathogenic bacteria, *Anaerococcus*, *Lysobacter*, *Mycoplasma*, *Peptoniphilus*, *Sneathia*, and *Prevotella* were more common, with higher copy numbers in the TV group. Conclusions: The data outlined the overall structure of vaginal communities, indicating that BV and TV were touching related to a sharp increase in the rich taxonomy and diversity of vaginal microbiota. VVC group presented a lower variety, with a significantly high abundance of *Lactobacillus.*

Keywords: Bacterial vaginosis, candida vaginitis, diversity, next-generation sequencing 16S rRNA gene (16SrDNA), trichomonas vaginalis, vaginal microbiome

Introduction

The vaginal microbiota, one of the important human-microbial habitats, includes a community of microbes with moderate diversity and has an interaction in the vaginal health, yet their correlation and the role of microbiota in health had been just recently confirmed. Studies have revealed that the microbe disruption results in increased susceptibility to various infectious diseases and adverse pregnancy outcomes [1-4]. 75% of women will undergo vaginitis at least once in their life [5]. Most experts believe that 90% of vaginitis cases are subsequently induced by bacterial vaginosis (BV), *Candida* vaginitis (VVC), and *Trichomonas* vaginalis (TV) [6-10]. They affect millions of women every year and are closely correlated with several adverse effects, containing preterm labor and delivery pelvic inflammatory disease [11-13], postabortal endometritis [14], etc., such as Neisseria gonorrhoeae, Chlamydia trachomatis, HPV, HSV-2 and HIV-1 [15-20].

The diagnosis of vaginitis mainly depends on the typical clinical symptoms, signs, and microscopic examination of vaginal discharge. Vaginal microscopy is easy to operate at a low cost. However, in practice, its accuracy is often susceptible to multiple factors, such as technical and subjective issues. In comparison, bacterial culture for the detection of bacteria flora

Table 1. The general data of the four groups

	NC group (n=10)	BV group (n=9)	VVC group (n=8)	TV group (n=4)	P
Age ($\bar{x}$ ± s, year)	32.39±7.20	30.19±6.37	34.18±8.24	35.14±9.03	0.629
BMI ($\bar{x}$ ± s, kg/m^2)	21.97±4.29	20.85±4.84	22.11±5.25	20.36±6.46	0.932
Duration ($\bar{x}$ ± s, month)	4.67±1.29	4.16±1.36	4.62±1.82	4.48±2.04	0.897
Delivery					0.507
Yes	8	6	7	4	
No	2	3	1	0	

is more accurate. Yet, this method is proven to be time-consuming and expensive, which limits its use in in-vitro diagnostic. Next-generation sequencing (NGS) techniques are the latest development in microbial community determination, which has significant improvement in the efficiency of vaginal microbiota research and achieves the high-throughput analysis of hundreds and thousands of samples with more detailed taxonomic information about the microbes [21-23]. This further our understanding that vaginal microbiota and the longitudinal changes occur in both healthy women and patients with vaginitis [24-26]. In addition, a great many molecular biological techniques based on 16SrDNA gene sequence diversity have been developed to explore the microbial community of the vaginal bacterial ecosystem.

Material and methods

Materials

In this experiment, 10 BV, 9 VVC, 8 TV patients, and 4 non-infected healthy women in the First Affiliated Hospital of Baotou Medical College in China from February to June 2015 were recruited. Ethics Committees of the First Affiliated Hospital of Baotou Medical College authorized the institutional review board approvals for the study. There was no significant difference in general data between the four groups. As shown in **Table 1**.

Inclusion and exclusion criteria

Inclusion criteria: 1) Patients with vaginitis confirmed by clinical and laboratory pathogenic examination [27, 28] before the test; 2) With follow-up conditions, good compliance and signed informed consent of volunteers; 3) 18-50 years of age, sexual life history, menstrual period; 4) Patients who were not treated with any oral medication in the last one month; 5) Patients who were not treated with any external drugs in the last two weeks; 6) The pregnancy test was negative in women of childbearing age, during the test can adhere to contraception and condom use in life.

Exclusion criteria: 1) Participants who were under 18 years old or above 55 years old; 2) Participants with pregnancy or diabetes; 3) Participants taking antibiotics or vaginal antimicrobials (orally or topically used to the vulvar/vaginal area) in the previous month; 4) Participants during their period or with menoxenia; 5) Participants with famous active co-infection with Chlamydia and Neisseria gonorrhoeae; 6) Participants with clinically apparent herpes simplex infection.

DNA extraction and MiSeq sequencing of 16S gene amplicons

DNA was purified according to standard methods in the reference [29]. Firstly, we used a NanoDrop Spectrophotometer and agarose gel electrophoresis to examine the DNA density and quality. Next, we diluted DNA extraction to 2 ng/μL and then stored it at -20°C for the use of downstream universal primers 5'-GTACTCCTACGGGAGGCAGCA-3' and 5'-GTGGACTACHVGGGTWTCTAAT-3'. In addition, 8 nt barcodes were used to amplify the V3V4 hypervariable areas of 16S rRNA genes by MiSeq sequencer [30, 31]. The PCR mixture (25 μL) was composed of 1× PCR buffer, 1.5 mM $MgCl_2$, 0.4 μM deoxyribonucleoside triphosphate, 1.0 μM primer, and 1 U of TransStart FastPfu DNA Polymerase (TransGen, China) and 4 ng genomic DNA. The PCR amplification steps are as follows: Firstly, 3 minutes of denaturation is performed at 94°C, next 23 cycles of 94°C for 30 seconds, next cooled to 60°C for 40 seconds, and up to 72°C for 60 seconds, and extension is the last stage when the sample is again subjected to 72°C for 10 minutes. Each sample received three PCR, and

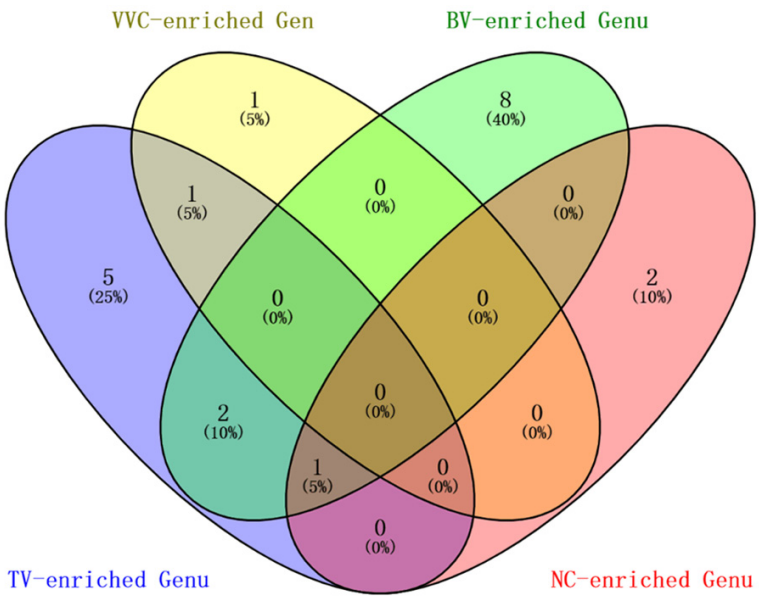

Figure 1. The distribution of enriched genus.

then it was combined together after amplifications. Next PCR products were given 1.0% agarose gel electrophoresis, and then we excised and purified the band with a correct size based on Gel Extraction Kit (Omega Bio-Tek, USA) and quantified it with the qubit. And all samples were pooled together with an equal molar amount. Following the instruction of the manufacturer, we prepared the sequencing library with TruSeq DNA Kit. According to the Illumina library preparation protocols, we diluted, denatured, and re-diluted the purified library, and thence mixed it with PhiX, which was equivalent to 30% of the final DNA amount, and next applied to an Illumina Miseq system with the Reagent Kit v3 (600 cycles). Based on the quantitative insights into microbial ecology (QIIME) pipeline [32], the original sequences were to link reads into tags according to the overlapped, then we separated reads in each sample with barcodes and removed low-quality reads. Then we gathered the processed tags and allocated the operational taxonomic units (OTUs) to taxa through matching in the Greengenes database [33].

Results

The quality of sequencing

After filtrating the low-quality reads and removing chimeras, we gained 1,108,830 high-quality reads, with 11,923 reads each sample. 10696 OTU were determined from the 93 samples. After the removal of OTU singletons (the number of the tag to 1 OTU) and chimeric sequence, the OTU number is 4533, and then OTU number of samples after sample dilution was 3030. The sequence length distribution was between 400 bp-440 bp, and their average length was 420 bp after managing the primers.

Microbiome diversity of different groups

At the genus level, these four groups differentiated each other. As shown in **Figure 1**, a total of 20 species of bacteria were found in different distribution profiles. The BV group not only possessed the most species but also had eight exclusive genera, respectively, *Arcanobacterium*, *Mobiluncus, Clostridium*, *Moryella*, *Shuttleworthia*, *Dialister, Bulleidia,* and *Megasphaera.* Five exclusive species, including *Gemella*, *Parvimonas*, *Peptoniphilus*, *Lysobacter,* and *Mycoplasma*, were found in TV group, which is second to BV group. *Varibaculum* and *Bacillus* were the two particular species of bacteria in NC group, and *Lactobacillus* is only one genus of bacteria found in VVC group.

Discussion

In general, a shift in microbial composition has a crucial effect throughout the progression of urogenital diseases. We have demonstrated that TV, BV, and VVC were correlated with changes in the vaginal microbiota compositions, and most of which were obvious at high taxonomic level (phylum) and even genus level. The composition and relative abundance of the vaginal microbiota by phylum did bot contribute to the etiology of BV, VVC, and TV. However, it exposed the holistic structure of the vaginal microbiota. Among the eight phylum in the vaginal ecosystem, *Firmicutes and Actinobacteria* comprised most of the vaginal microbiota in four groups, especially more prominent in the VVC group. *Proteobacteria* takes

up a considerable proportion of the population in the healthy group, while *Bacteroidetes* and *TM7* were closely correlated with BV, *Tenericutes*, *Fusobacterlia,* and *Bacteroidetes* were strongly associated with TV. In comparison, *Lactobacillus* typically shows a predominance in the vaginal microbiome of healthy women, similar to the data from Asian women in America [3, 4]. Although longitudinal studies on healthy women have discovered the fluctuations of the vaginal microbiome from a situation where *Lactobacillus* dominates to another structure, most of the snapshot materials follow the predominant pattern of *Lactobacillus* [34]. It is the main bacterial communities in the healthy vagina containing lactic acid bacteria that doesn't surprise us. Because these genera stay at a low vaginal pH via their metabolic activity and thus differ in the colonization resistance that protects from the invasion of obvious pathogens or from the overgrowth and dominance of potentially pathogenic species among the normal microbiota.

BV appears to be a polymicrobial process with interrelated organisms, which can lead to a common outcome though the changing patterns, were not always the same for all samples. In the process of normally developing into BV, the vaginal pathogenic bacteria substitutes the healthy vaginal microbiota bit by bit, which is seen through the decrease in the *Lactobacillus spp.* and other facultative or anaerobic species [35]. In this study, we also demonstrated that *Lactobacillus* decreases while the vaginal pathogenic bacteria increases, such as *Anaerococcus*, *Arcanobacterium*, *Megasphaera*, *Sneathia,* and *Prevotella*. The tendency of the microbial ecosystem resulted in changes from eubiosis to dysbiosis during the advent of BV. Although previous studies have discovered that *Gardnerella vaginalis* (belonging to *Actinobacteria*) was a good marker for BV [36-38], we did not find its presence in the BV samples and only detected it in very low abundance in the non-BV samples. The low detection rate of *Gardnerella vaginalis* may be attributed to the depth of the Illumina Miseq sequencing. In addition, some studies also discovered this species in subjects who didn't have BV with a low abundance level [39]. Whether *Gardnerella vaginalis* is suitable for BV diagnosis markers is also up for debate. Consistent with other studies' findings, *Mobiluncus* was observed in vaginal bacterial communities only in the presence of BV, which was also highly resistant to metronidazole [40, 41]. *Prevotella* (belonging to *Bacteroidetes,* predominant microbiota in the complex vaginal communities of BV) and S*neathia* (belonging to *Fusobacteria,* a lactic acid-producer) also showed a strong association with BV.

Recent evidence has proved that TV infection is changed by the microbiome of women [42, 43] and TV treatment is altered through using the microbiome [44]. The cluster analysis was performed to further visualize the association between TV and the composition of vaginal microbiota. Among vaginal pathogenic bacteria, *Anaerococcus*, *Lysobacter*, *Mycoplasma*, *Peptoniphilus*, *Sneathia*, and *Prevotella* were more ordinary, with higher copy numbers in the TV group ($P<0.05$). TV has a visually higher average of 14.5% *Lactobacillus* compared with BV but lower compared with NC. However, the distribution of *Anaerococcus*, *Sneathia,* and *Prevotella* are like the BV microbiota indicating that the two types vaginal infections frequently occur as co-infections among women [45-48]. *Mycoplasma* is a well-recognized component of bacterial vaginosis microbiota and quantitative *Mycoplasma* nucleic acid amplification assays are predictive of bacterial vaginosis [49]. According to our data, we would think that *Mycoplasma* was in high abundance of in TV instead of BV. The heat map from cluster analysis proved that the vaginal flora of TV was characterized by high abundance of *Mycoplasma* and relatively few *Lactobacillus*, indicating that TV had a direct impact on the microbial environment and confirmed the potential significance in the interactions between TV and vaginal microbiota. In vitro studies have demonstrated that *Mycoplasma* is taken up by TV and is able to survive within cytoplasmic vacuoles [50, 51]. Moreover, *Mycoplasma* imposes significant influence on the metabolism of TV [52] and may increase its pathogenicity [53]. Considering the strength of the association between *Mycoplasma* and TV, the fact is that *Mycoplasma* is an obligate symbiont. Another organism closely correlated here with TV is *Parvimonas,* which is a common oral pathogen associated with dental root canal infections [54, 55]. Thus, there exist clear differences in the com-

position of vaginal bacterial communities between TV women and TV-uninfected women.

There is controversy about the effect of vaginal microbiota in VVC in the references. VVC is an ordinary adverse reaction caused by BV treatment with antibiotics, indicating that the vaginal microbiota might be relevant to the yeast colonization [56]. In our studies, two distinct points were found in comparison with other references [57, 58]: with a significant high abundance of *Lactobacillus*, VVC samples presented a few community structures. Two kinds of OTUs, including *Aerococcus* and *Lactobacillus* are known to be associated with VVC. The predominant bacterial population in VVC vagina was *Lactobacillus* at the level of the genus. In comparison, other studies have found that there was abundant *Lactobacillus spp*. in VVC, presenting a significant difference in the vaginal microbiomes between VVC patients and healthy controls. With the consideration of the depletion of *Lactobacillus* in BV and TV patients, Candida infection may create a condition that was beneficial to *Lactobacillus* growth. Our present findings showed the likelihood of symptomatic VVC might be significantly increased when there was a decrease in the diversity of vaginal flora and a concomitant emergence of a lot of *lactobacilli*. Whether or not *lactobacilli* increase the risk of VVC [4-59, 60] is still in debating.

Conclusion

In summary, our results support that there are more diverse vaginal microbiota in TV or BV participants than we expected before, with a relatively low abundance of *Lactobacillus*. But to our surprise, the VVC samples present fewer community structure, with a significant high abundance of *Lactobacillus*. Specifically, the results indicate that there isn't any decrease in the proportion of *lactobacilli* in the vaginal communities of women with VVC, suggesting that indigenous vaginal bacterial species like *Lactobacillus* probably are not crucial factors protecting from Candida infections. Furthermore, there may be a significant increase in the likelihood of symptomatic VVC when the decrease in the diversity of vaginal flora and the concomitant emergence of a lot of *lactobacilli*. In comparison, our results typically present that Lactobacillus shows a predominance in the vaginal microbiome of healthy women. However, our study still had some limitations. First, it is a cross-sectional study, so longitudinal cohort studies will be required to address the microbiota profile about low genital infection disease. Second, we haven't explored dynamics of vaginal bacterial communities and its diversity in the reconstruction of vaginal microbiota after effective treatment. It is necessary to further study the interaction between the vaginal microbiota and the host, especially those bacteria found in our study with low abundance, so as to have an extensive understanding of the ecological role of the complex vaginal bacterial communities. To better know of the interactions of host and bacteria in the vagina and make a customized treatment for vaginal infectious diseases, high-throughput sequencing techniques are used to carry out longitudinal samples before and after treatment in further studies.

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Disclosure of conflict of interest

None.

Abbreviations

BV, Bacterial vaginosis; VVC, Candida vaginitis; TV, Trichomonas vaginalis; NGS, Next-generation sequencing.

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