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Research paper

Identification of vaginal bacteria diversity and it's association with clinically diagnosed bacterial vaginosis by denaturing gradient gel electrophoresis and correspondence analysis

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ABSTRACT

Bacterial vaginosis (BV) is a common complex associated with numerous adverse health outcomes, affecting women of different ages throughout the world. The etiology of BV remains poorly understood due to the difficulty of establishing a molecular genetic criterion to recognize the vaginal microbiota of BV-positive women from that of the normal women. We used techniques of broad-range PCR-DGGE and gel imaging analysis system cooperated with 16S rRNA gene sequencing and statistical analysis to investigate the community structure of the healthy and BV-affected vaginal microbial ecosystems. The community of vaginal bacteria detected in subjects with BV was far more luxuriant and diverse than in subjects without BV. The mean number of microbial species in 128 BV-positive women was nearly two times greater than in 68 subjects without BV(4.05 ± 1.96 versus 2.59 ± 1.14). Our sequencing efforts yielded many novel phylotypes (198 of our sequences represented 59 species), including several novel BV-associated bacteria (BVAB) and many belonging to opportunistic infections, which remain inexplicable for their roles in determining the health condition of vaginal microflora. This study identifies *Algoriphagus aquatilis*, *Atopobium vaginae*, *Burkholderia fungorum*, *Megasphaera genomosp* species as indicators to BV and subjects with BV harbor particularly taxon-rich and diverse bacterial communities. Maybe *Bifidobacterium*, *Staphylococcus* or even more alien species are commensal creatures in normal vaginal microbiota.

1. Introduction

Bacterial vaginosis (BV) is a common vaginal disorder characterized by an alteration of the normal vaginal bacterial microbiota to a heterogeneous state containing a complex population of anaerobic and microaerophilic organisms. BV is associated with sexually transmitted infections and adverse pregnancy outcomes such as low birth weight of foetus, premature birth, neonatal death and secondary infection after delivery and is also the risk factor for premature rupture of fetal membranes (PROM) (Goldenberg et al., 2008). Antibiotics treatment of the condition used to failure due to the uncertain variation of BV-associated bacteria (BVAB) (Beigi et al., 2004; Ferris et al., 2004). Many BV associated bacteria are resistant to culture and (or) difficult to detect by traditional methods, which thwart the exploration of the pathogenesis of BV. So far, there is a lack of effective prevention and treatment measures.

According to Nugent's classification (Nugent et al., 1991), a score ranged from 7 to 10 is considered as BV, a clinical condition dominated by the morphological identification of Gram-negative and-positive bacteria, without evidence of *Lactobacillus* morphotypes. In contrast, the score from 0 to 3 is considered an undisturbed vaginal microflora dominated by the *Lactobacillus* genus, identified as the principal Gram-positive rods bacteria.

In general, race (nationality) (Ness et al., 2003; Uscher-Pines et al., 2009), behavioral habits (Bradshaw et al., 2013), personal hygiene status (Brotman et al., 2008), and so on have been considered as causes of BV. Although there is agreement in the literature that no single agent likely causes BV, there is no consensus about what constitutes a pathogenic bacterial community in this syndrome. Comprehensive cultivation-independent comparisons of the vaginal bacterial communities between subjects with and without clinically defined BV have been rare.

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With the development and application of culture-independent nucleic acid amplification test (NAAT) in recent years, it is possible to explore the structure and dynamic shift of microbiota (Larin et al., 2007; Burton and Reid, 2002; Zozaya-Hinchliffe et al., 2010; Ravel et al., 2011). Molecular methods have identified in the vagina of healthy, nonpregnant women the *Lactobacillus* genus living with a spectrum of bacteria including *Gardnerella*, *Atopobium*, *Eggerthella*, *Megasphaera*, *Leptotrichia*, *Prevotella*, *Enterococcus*, *Bifidobacterium*, *Staphylococcus*, *Corynebacterium*, *Streptococcus*, *Bacterioides*, *Mycoplasma*, *Escherichia*, *Peptostreptococcus*, *Ureaplasma*, *Veillonela*, and *Candida* species (Zhou et al., 2004; Ling et al., 2013; Hyman et al., 2005; Vitali et al., 2007; Nam et al., 2007). However, at present the spectrum of bacterial species that are correspondent to clinical diagnosis, i.e. the Nugent's score is not well defined.

In this study, we investigated the composition of BVAB in countryside women of reproductive age. Women with and without BV was diagnosed by Nugent's criteria and recruited as the research subject and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) and DNA sequencing techniques were used to analysis the genetic information of 16S rRNA genes (16S rDNA) of vaginal bacteria. The sequence information of 16S rDNA of every detected bacterium was identified with gene bank data. In addition, DGGE Gel Image System was used to calculate the relative abundance of each bacterial species to the whole microbial population of one sample (vaginal swab). Eventually, appropriate statistical method was used to analyze the relationship between BV and bacterial composition, as well as the relationship between BV and other risk factors.

2. Materials & methods

2.1. Ethics statement

All participants enrolled in this study signed written informed consent to their participation. The study protocol and consent form were approved by the ethic review board at Institute of Basic Medicine of Shandong Academy of Medical Sciences.

2.2. Study subjects

196 asymptomatic or symptomatic premenopausal women with a median age of 36 (ranges 18–48) were enrolled from Qilu hospital, Shandong University. 128 women were diagnosed as BV due to their Nugent scores ranging from 7 to 10, whereas 68 were defined as normal that having scores of 0–3. In order to confirm the outcome of Nugent scores criterion, a commercially available test kit (supplied by Sciarray Biotech Co., ShenZhen city, China) was used. This test kit is based on the principle that lactic acid and hydroperoxide are poorly produced by most BV-related organisms and the pH value of vaginal leakage is dramatically different between BV(usually pH ≥ 4.5) and normal(usually pH < 4.5) women. All the women were in good general health and were not undergoing current antibiotic treatment. After thoroughly wiping, the swabs were collected from the posterior fornix of the vaginal tract from 196 women with and without BV, packaged and placed at −40 °C for bacterial DNA extraction and subsequent experiment.

2.3. Extraction of bacterial DNA from vaginal swabs

It is so pivotal for sensitively detecting all the ingredients of the vaginal biota that we adopted an effective DNA extraction method. Briefly, swabs were vigorously agitated in 1 mL of physiological saline solution to disperse cells into a liquid phase. The cells were precipitated in Eppendorf tubes by centrifugation at 10,000*g* for 5 min, washed by re-suspending cells in PBS(pH = 6.8) and centrifuged again at 13,000 g for 3 min. The sediments were re-suspended in 200 μL lysis matrix (mainly consisted of SDS, proteinase K), and the mixture were incubated for 1–2 h in a water bath at 55 °C. The sample was vortexed for 10 s and boiled at 100 °C for 8 min and was vortexed again for 10 s and centrifuged at 13,000*g* for 3 min. The supernatant containing DNA template was stored at −20 °C or used directly for PCR process.

2.4. PCR amplification of the DNA samples

The amplification reactions of the DNA sample were carried out in single 0.2 mL PCR tubes (RNase/DNase/pyrogen free) using a thermocycler (Bio-Rad Laboratories). Each PCR reaction system consisted of 5.0 μL of 10 × buffer (No $MgCl_2$, 10 mM Tris-HCl, and 50 mM KCl), 2.5 μL of $MgCl_2$ (50 mM), 0.5 μL dNTPs (10 mM each), 1.25 μL of glycerol (80%) (Invitrogen™, Life Technologies), 4.0 μL of Bovine serum albumin (BSA) (10 mg/mL) (Invitrogen™, Life Technologies), 50pmoles/μL of primer HDA-1 GC (5′-CGC CCG GGG CGCGCC CCG GGC GGG GCG GGG GCA CGG GGGGAC TCC TAC GGG AGG CAG CAG-3′) and primer HDA-2 (5′-GTA TTACCG CGG CTG CTG GCA-3′) (Invitrogen™, Life Technologies), 0.2 μL of Platinum® Taq DNA polymerase (5 U/μL) (Invitrogen™, Life Technologies), 2.0 μL of the DNA sample, and sterile water to a volume of 50 μL. PCR amplification involved an initial DNA denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °Cfor 30 s, annealing at 60 °C for 1 min and elongation at 72 °C for 1 min, which was followed by a final extension at 72 °C for 7 min. To confirm amplicon production, the mixture (5 μL PCR product and 2 μL of loading buffer) was analyzed by electrophoresis (Bio-Rad Laboratories) using 1.5% Ultrapure™ Agarose (Invitrogen, Life Technologies) gels at 100 V for 45 min, followed by staining with 1% solution of ethidium bromide (50 μL/L). Gels were visualized and recorded by ultraviolet transillumination.

2.5. DGGE (denaturing gradient gel electrophoresis)

Preparation of gel gradients and electrophoresis was carried out according to the manufacturer's instructions for the D-Code™ Universal Mutation Detection System (Bio-Rad Laboratories). The concentrations of the polyacrylamide, gradient denaturant and Tris-acetate buffer (40 mM Tris base, 20 mM glacial acetic acid, 1 mM EDTA [pH 8.0],TAE) were 8%, 30%–60% (7 M urea and 40% deionized formamide were considered to be 100% denaturant), and 50×, respectively. 10 μL of amplified PCR products were mixed with 20 μL of 2 × loading buffer (0.25 mL of bromophenol blue [2%, Sigma], 0.25 mL of xylene cyanol [2%, Sigma], 7 mL of glycerol, and 2.5 mL of distilled H_2O) and loaded into the wells.

Gels were run at 130 V and 65 °C for 3.5–4.0 h in 1 × TAE until the second dye front (xylene cyanol) approached the end of the gel. After electrophoresis, gels were removed and allowed to cool under flowing tap water before the removal of the glass-plate sandwich. The gel was visualized by silver staining and the gel images were analyzed and calculated using a gel imaging and analysis system (chemID™, Saizhi Co, BeiJing city, China). Densitometric measurement of each bands were performed by ChampGel Image System, version 3.0 (Saizhi Co, BeiJing City, China) to generate the ratio of the peak height of a potential band to the sum of the peak heights in the banding profile of every electrophoretic lane, Which corresponded to the relative amount(proportion) of one microbial species to the sum of species in a certain sample.

2.6. Band excision from denaturing gradient gels

DGGE fragment bands were excised from the gels with a sterile scalpel and placed into single 1 mL Eppendorf tubes. Cut bands were washed in 1 × PCR buffer and incubated in 35 μL of the same buffer overnight at 4 °C.