Comparison of culture-dependent and culture-independent molecular methods for characterization of vaginal microflora

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Abstract

Purpose. To date, molecular methods that circumvent the limitations of traditional culture methods have not been used to describe the vaginal microflora in India. Here, we compared culture and culture-independent molecular methods in characterizing the vaginal microbiota in Indian women.

Methodology. Culture methods involved traditional cultivation on Rogosa and sheep blood agar, whereas culture-independent methods bypassed a culturing step by performing broadrange 16S rDNA PCR on DNA isolated directly from vaginal swabs.

Results. A total of 13 women were included in the study, of which five were characterized as healthy, two were bacterial vaginosis intermediate and six were bacterial vaginosis positive according to Nugent scoring. Lactobacillus jensenii was detected most frequently when using culture methods. On the other hand, Lactobacillus iners, which was not detected by culture methods, was the most common Lactobacillus sp. detected using cultivation-independent methods.

Conclusion. We found little overlap between the species found using cultivation-dependent and cultivation-independent methods. Rather, culture-dependent and culture-independent methods were found to be complementary in describing the vaginal microflora among South Indian women. Culture-independent methods were found to be superior in detecting clinically relevant vaginal flora.

INTRODUCTION

The vaginal microbiome in healthy women is predominately lactobacilli, which are thought to be important for maintaining normal vaginal environment and protecting against infections [1]. Disturbance of the vaginal microecosystem causes bacterial vaginosis (BV), a clinical condition characterized by a decrease in lactobacilli and the overgrowth of anaerobic and facultative Gram-negative and Gram-variable bacteria. BV is a risk factor for sexually transmitted infections including HIV [2, 3].

While vaginal microflora were traditionally characterized using cultivation methods, today, cultivation-independent molecular approaches are favored because certain vaginal microflora are not amenable to culturing [4–7]. Thus, culture-independent methods have demonstrated greater vaginal microbial diversity than previously recognized and have identified under-documented species such as Lactobacillus iners and Atopobium vaginae as important constituents of the vaginal microbiome worldwide [7–16].

We have previously used culture methods to characterize the vaginal microbiome in Mysore [17, 18]. In this study, we have compared traditional cultivation and cultivation-independent molecular methods side by side. Unlike cultivation processing, cultivation-independent methods circumvented culturing vaginal swabs on Rogosa and sheep blood agar plates by directly extracting total DNA from swabs before completing 16S rDNA PCR and sequencing. The use of both techniques together on the same cohort of women also allows a more comprehensive description and comparison of the vaginal microbiome in South India. To our knowledge, this is the first Indian study to characterize vaginal microflora using both culture-dependent and culture-independent processes.
METHODS

Between August 2014 and May 2015, a cross-sectional study was conducted among women seeking healthcare services at the Prerana Health Clinic. Eligibility criteria for the study were the same as used previously [18]. Briefly, eligible women had to be between 18 and 35 years of age and sexually active in the 30 days prior to enrolment with ability to provide informed consent. Pregnant or menstruating women; those taking immunosuppressants, antibiotics or antifungals; or those who had had reproductive tract infections within the past 30 days were excluded. Informed consent was obtained from each participant in the local language of Kannada. The study was reviewed and approved by the Institutional Review Boards at Florida International University and Public Health Research Institute of India.

Study participants were asked to provide a self-collected vaginal swab to diagnose BV using Nugent scoring [19]. Under the Nugent scoring system, a standardized 0–10 point scoring system, a score of 0–3 was deemed to be ‘healthy’, 4–6 ‘intermediate’ and a score of 7–10 considered ‘BV’. Amsel criteria were also assessed in order to corroborate the classification of healthy and BV-positive women. According to Amsel criteria, women are classified as BV positive based on the presence of at least three of the following four characteristics: milky white vaginal discharge, clue cells on wet mount microscopy, vaginal fluid pH >4.5 and a fishy odor upon addition of potassium hydroxide (KOH) [20]. The healthy women according to Nugent score had none or no more than one of the Amsel criteria characteristics.

Three additional swabs were collected from each participant by a physician from the posterior fornix of the vagina. For culture-dependent processing, two swabs were immediately placed in BBL Port-A-Cul transport tubes (Becton, Dickinson and Company) and plated onto Rogosa and sheep blood agar within 12 h. Colonies were grown anaerobically and total DNA was isolated using the QIAamp Fast DNA Stool Mini Kit (Qiagen). Briefly, the manufacturer’s protocol was followed with a 95 °C lysis step and the following volume modifications: the entire pellet was completely resuspended in 250 µl InhibitEX buffer; to this resuspension, 250 µl buffer AL and 250 µl 100% ethanol were added; finally, DNA was eluted in 50 µl of buffer ATE. Total DNA was stored at −20 °C. 16S rDNA PCR analysis was conducted using 1 µl of the total DNA and the same primers and protocol as for the culture-dependent processing. PCR products were then cloned into the pCR 2.1 vector (Invitrogen) using the manufacturer’s protocol, and 2 µl of the cloning reaction was transformed into DH5α Escherichia coli chemically competent cells (Invitrogen). Blue–white screening was used to choose positive transformants, and colony PCR was performed using the M13F/R primer set to assess the presence of the 16S rDNA insert. Ten clones containing inserts were isolated from each woman. Next, HaeIII restriction digestion analysis was performed on colony PCR products and analyzed on a 3 % agarose gel to select clones with inserts from different species. At least four clones from each woman were selected for analysis; plasmid DNA was prepared and sequenced with the M13F primer (Invitrogen) on an ABI 3730xl DNA Sequencing system at SciGenom Labs. As with culture-dependent methods, sequences were matched on NCBI BLAST based on the closest BLAST identity of 80 % and match length of 500 bp.

Non-Lactobacillus species were classified according to whether they were significantly associated with BV, based on a previous analysis by Srinivasan et al. [21]. Bacterial taxa in the present study were classified as either ‘BV associated’ (P<0.05) or ‘BV unassociated’ (P>0.05). A single species that was taxonomically unannotated in NCBI was classified as ‘other’.

For culture-independent processing, the third vaginal swab was immediately placed in a conical tube containing 5 ml of sterile PBS (pH 7.0). The tube was vortexed vigorously for 3 min, the swab removed and the tube spun down in a clinical centrifuge at maximum speed for 5 min. The supernatant was removed, and the cell pellet was kept on ice for no longer than 1 h until it was transported back to the laboratory and stored at −80 °C. Cell pellets were thawed on ice, and total DNA was isolated using the QIAamp Fast DNA Stool Mini Kit (Qiagen). Briefly, the manufacturer’s protocol was followed with a 95 °C lysis step and the following volume modifications: the entire pellet was completely resuspended in 250 µl InhibitEX buffer; to this resuspension, 250 µl buffer AL and 250 µl 100% ethanol were added; finally, DNA was eluted in 50 µl of buffer ATE. Total DNA was stored at −20 °C. 16S rDNA PCR analysis was conducted using 1 µl of the total DNA and the same primers and protocol as for the culture-dependent processing. PCR products were then cloned into the pCR 2.1 vector (Invitrogen) using the manufacturer’s protocol, and 2 µl of the cloning reaction was transformed into DH5α Escherichia coli chemically competent cells (Invitrogen). Blue–white screening was used to choose positive transformants, and colony PCR was performed using the M13F/R primer set to assess the presence of the 16S rDNA insert. Ten clones containing inserts were isolated from each woman. Next, HaeIII restriction digestion analysis was performed on colony PCR products and analyzed on a 3 % agarose gel to select clones with inserts from different species. At least four clones from each woman were selected for analysis; plasmid DNA was prepared and sequenced with the M13F primer (Invitrogen) on an ABI 3730xl DNA Sequencing system at SciGenom Labs. As with culture-dependent methods, sequences were matched on NCBI BLAST based on the closest BLAST identity of 80 % and match length of 500 bp.

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RESULTS

Twenty-six women were initially enrolled in the study (Fig. 1). Samples from 13 women failed during culture-dependent processing due to lack of colony growth or failure to amplify a 16S PCR product. Full analysis was completed for samples from 13 women using both culture-dependent and culture-independent methods. The average age of the women enrolled in the study was 28.5 years (SD, ±4.8). All 13 participants were Hindu and married.

Ultimately, five sets of samples (38.5%) were obtained from healthy women, two (15.4%) from intermediate and six (46.2%) from BV-positive women (Fig. 1). A total of 47 species, representing 26 unique species, were obtained using culture-dependent methods, and a total of 51 species, representing 27 distinct species, were isolated using culture-independent methods (Table 1). The list of bacterial species isolated from each participant using both methods is shown in Table S1 (available in the online Supplementary Material).

Culture methods found eight distinct Lactobacillus species among the 13 women (Table 1). Lactobacillus jensenii was the most common species, found in four (31%) women (Table 2, Fig. 2). Lactobacillus gasseri and Lactobacillus reuteri were isolated from two (15%) women each, while Lactobacillus crispatus, Lactobacillus mucosae, Lactobacillus fermentum, Lactobacillus ruminis and Lactobacillus coleohominis were isolated from one (8%) woman each. Cultivation-independent methods isolated a different, but overlapping set of nine Lactobacillus species. (Tables 1 and 2).

Seven Lactobacillus species were found using both techniques (Table 2, Fig. 2). L. ruminis was only found with culture-dependent techniques, while L. iners and L. johnsonii were only found with culture-independent techniques. The majority (76%) of Lactobacillus species were isolated from women who were healthy or had intermediate stage flora (Tables S1 and 2).

In total, 34 non-Lactobacillus species were found using culture-dependent methods, of which 18 were unique species (Tables S1 and 1). A set of 31 non-Lactobacillus species were found using culture-independent methods, of which 18 were also unique species. Using culture-dependent techniques, 85% of non-Lactobacillus species isolated were non-pathogenic species such as Staphylococcus epidermidis and Enterococcus faecalis (Table 3). Only 12% of non-Lactobacillus species isolated using culture methods were correlated with a positive BV status. Conversely, approximately 77% of non-Lactobacillus species isolated using culture-independent methods were known to be associated with BV, such as Atopobium vaginae, Sneathia sanguinegens and Prevotella spp. Accordingly, these species were generally isolated from BV-positive women. Only 23% of non-Lactobacillus species found using culture-independent methods were not associated with BV.

Table 1. Summary of species isolated among women of reproductive age in Mysore, India

The table shows the total numbers of species, unique species, Lactobacillus and non-Lactobacillus species isolated using culture-dependent and culture-independent techniques. It also shows the number of species found from each participant that were found using both culture-dependent and culture-independent methods (overlapping species).

<table>
<thead>
<tr>
<th>Patient</th>
<th>BV status</th>
<th>Culture dependent</th>
<th>Culture independent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lacto species</td>
<td>Non-lacto species</td>
<td>Total species</td>
</tr>
<tr>
<td>1</td>
<td>Neg.</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>Pos.</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>Pos.</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>Int.</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>Neg.</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>Pos.</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>Int.</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
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<td>2</td>
</tr>
<tr>
<td>9</td>
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</tr>
<tr>
<td>13</td>
<td>Pos.</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
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<td>47</td>
</tr>
<tr>
<td>Unique</td>
<td>8</td>
<td>18</td>
<td>26</td>
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</table>

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We found little overlap between the species found in each individual woman using culture-dependent and culture-independent methods. In all, of the 47 species isolated using culture-dependent methods and the 51 species isolated using culture-independent methods, only five species were found from the same woman using both techniques (Table 1). Only four of the 13 women (31%) had at least one species found using both techniques. Moreover, three of these four women only had one species that was isolated using both techniques.

**DISCUSSION**

In comparing culture-dependent and molecular culture-independent methods side by side, we did not find one technique to be clearly superior over the other; when used in parallel, the two techniques isolated different, largely non-overlapping sets of vaginal microbial species from the same women. Nevertheless, we have demonstrated some clear strengths of molecular cultivation-independent approaches. For one, we were able to identify *L. iners* as a prevalent vaginal microbe in healthy Indian women. To date, *L. iners* has never been detected in the Indian vaginal microflora [17, 22]. Here, we not only identified *L. iners* in the vaginal microflora in South Indian women, but we found it to be the most prevalent species when using molecular approaches.

Another notable strength of culture-independent methods was that it was better at detecting clinically relevant non-*Lactobacillus* species known to be associated with BV than traditional culture methods. This is consistent with previous research that reports that culture methods select for abundant, rapidly growing, aerobic microbes such as *Staphylococcus epidermis* [23], while under-detecting species such as *Atopobium vaginae* and *Leptotrichia* spp. [7, 24, 25].

As a pilot study, one limitation of our study is the relatively small size (n=13). As such, we did not have statistical power to measure the true prevalence of certain bacterial species. Since we sampled clones per sample, it is likely that many species were not found due to their relatively lower abundance. Finally, half of the samples were excluded either due to overlapping sets of vaginal microbial species from the same women. Nevertheless, we have demonstrated some clear strengths of molecular cultivation-independent approaches. For one, we were able to identify *L. iners* as a prevalent vaginal microbe in healthy Indian women. To date, *L. iners* has never been detected in the Indian vaginal microflora [17, 22]. Here, we not only identified *L. iners* in the vaginal microflora in South Indian women, but we found it to be the most prevalent species when using molecular approaches.

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to lack of growth on culture or failure during 16S rDNA PCR, which could be a source of potential bias in the study.

Despite these limitations, this study is the first to molecular methods to characterize the vaginal microflora among women of reproductive age in India. This direct comparison provides a rubric to gauge the representativeness of previous data. Our results show that, although previously unrecognized as a component of the vaginal microbiome in India, \textit{L. iners} is prevalent in the population. We have clearly demonstrated the utility of using molecular approaches to characterize vaginal microbial diversity over the traditional culturing methods previously used in this setting.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

References