Preliminary characterization of the normal microbiota of the human vulva using cultivation-independent methods

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The objective of this study was to perform a preliminary characterization of the microbial populations of the normal human vulva. Genomic DNA was isolated from samples of the labia majora and labia minora from four healthy women, and sequences of bacterial 16S rRNA genes in each were determined. The sequences were compared with those of known bacterial species to classify the numerically abundant populations in these communities. Even among this limited number of individuals, the microbiota of the human vulva was found to be quite diverse. Each woman had a distinctive microbiota and no single species was common to all women. The microbiota of the labia majora and labia minora differed, although both had appreciable numbers of lactobacilli and strict anaerobes. A greater diversity of populations inhabited the labia majora compared with the labia minora. The results indicated that the microbiota of the vulva includes populations known to be commensals of the microbiota of the skin, colon and vagina, and is much more complex than previously thought, suggesting that more extensive investigations are warranted.

INTRODUCTION

The human vulva consists of several distinct ecotopes that are defined by their physical and chemical characteristics. The physical characteristics include distinct histological architectures and anatomical proximity to the vagina, urethra, anus and Bartholin’s gland, whilst the chemical factors include the composition and amount of vaginal secretions, as well as contamination by urine and faecal material. As a result, the microbiota of the human vulva is complex and unique (Elsner & Maibach, 1990). Cultivation-dependent methods have been used previously to study the microbiology of the labia majora. The microbiota of the labia majora is characterized by micro-organisms that are either related to vaginal and urethral inhabitants (such as Lactobacillus spp.) or are common for intertriginous skin, including Gram-negative rods, non-pathogenic Neisseria, Gardnerella vaginalis and Staphylococcus aureus (Aly et al., 1979; Elsner & Maibach, 1990). Technical difficulties in the isolation of bacteria have confounded efforts to obtain a comprehensive understanding of the organisms present in various anatomical locations on the human body (Berg, 1999; Henderson et al., 1998; Hooper et al., 2001; Pace, 1997; Rappe & Giovannoni, 2003; Tlaskalova-Hogenova et al., 2004). This raises the possibility that organisms that normally reside in the human vulva were overlooked in previous studies that employed cultivation-dependent methods.

Micro-organisms present on the vulva of healthy women may have clinical significance because their presence may affect the proliferation of non-indigenous populations (Atassi et al., 2006; Reid & Burton, 2002), including those that play a crucial role in triggering various common diseases such as vulvovaginitis (Wilson, 2005), urinary tract infection (Reid, 1999), bartholinitis (Nakatsu et al., 2003; Tlaskalova-Hogenova et al., 2004) and abscesses of Bartholin’s gland (Tanaka et al., 2005). Vulvovaginitis is characterized by inflammation or infection of the vulva and vagina. It affects women of all ages and is very common (Makela et al., 2003; Merkley, 2005; Sobel, 2001, 2003; Wilson, 2005). It can be caused by bacteria, yeasts, viruses or parasites, including some that are responsible for sexually transmitted diseases (Makela et al., 2003). Vulvovaginal candidiasis, which is caused by species of Candida, is one of the most common forms of vulvovaginitis in women of all ages (Eschenbach, 2004; Sheary & Dayan, 2005; Sobel, 2004; Spence, 2003; Xu & Sobel, 2004). Approximately 75 % of women in the USA experience vulvovaginitis caused by Candida at some time during their reproductive years (Wilson, 2005), between 40 and 50 % of women have recurrent episodes (Sheary &
Dayan, 2005), and 5–8% experience chronic Candida infections (Eschenbach, 2004). As relatively little is known about the microbiology of the vulva, the aetiology and precipitating factors of vulvovaginitis are not well understood. Consequently, the treatment and management of this disease represent a large burden on the health-care system. An accurate understanding of the composition and ecology of the healthy vulvovaginal microbiota may provide insight into components of the microbiota that may reduce the risk of acquiring this and other diseases. However, to elucidate fully the potential protective effects of the healthy vulvovaginal microbiota would require comparisons between the healthy and compromised or diseased state, or between high-risk and low-risk groups.

The aim of this preliminary study was to investigate the microbial communities in the normal human vulva of four healthy adult women. Our approach utilized partial sequencing of 16S rRNA genes to identify bacterial populations that were abundant in the labia majora and the labia minora.

**METHODS**

**Clinical samples.** The study protocol and informed-consent documents were reviewed and approved by the Procter & Gamble Corporate Institutional Review Board. Five healthy white women, aged 28–44 years, were recruited at visit 1 to complete health questionnaires and provide written informed consent. During this visit, they received Olay Moisturizing Bar – Sensitive Skin (Procter & Gamble) for body cleansing and Always Ultra Thin (Procter & Gamble) catamenial pads, which were used for at least 1 month prior to the sampling visit in order to standardize the environment among the women. All of the women were in good general health, reported regular menstrual cycles, used the supplied cleansing bar among the women. All of the women were in good general health, reported regular menstrual cycles, used the supplied cleansing bar and Gamble) catamenial pads, which were used for at least 1 month prior to the sampling visit in order to standardize the environment among the women. 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Distance and clustering analyses were performed using PHYLIP using the neighbour-joining algorithm (Saitou & Nei, 1987). The Sequences were then clustered together based on their genetic distances the aligned sequences from about nt 65 to 450 in the Cantor, 1969) was used to calculate pair-wise genetic distances among.

The study of the human vulva by culture-independent methods resulted in the identification of many phylotypes not previously known to exist at this anatomical site. Previous culture-dependent studies of the vulva have shown that micrococci, α- and β-haemolytic streptococci, lipophilic diphtheroids, non-lipophilic diphtheroids, bacilli, lactobacilli, Gram-negative and Gram-positive rods, and both S. aureus and coagulase-negative staphylococci are present (Aly et al., 1979; Elsner & Maibach, 1990). Sequences with similarity to representatives from each of these groups of organisms were also found in our study, with the notable exception of S. aureus. We cannot explain the absence of S. aureus in our samples, but suspect that it may be due to the

Table 2. Phylotypes found in the clone libraries of vulvar samples from four healthy women

<table>
<thead>
<tr>
<th>Bacterial species*</th>
<th>W2 A (n=105)</th>
<th>B (n=101)</th>
<th>W3 A (n=101)</th>
<th>B (n=114)</th>
<th>W4 A (n=106)</th>
<th>B (n=87)</th>
<th>W5 A (n=153)</th>
<th>B (n=120)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus iners</td>
<td>70.5 (0.4)</td>
<td>4.4 (0.2)</td>
<td>96.2 (0.2)</td>
<td>22.8 (0.3)</td>
<td>32.0 (0.2)</td>
<td>7.2 (0.5)</td>
<td>0.9 (5.3)</td>
<td>28.3 (0.2)</td>
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<tr>
<td>Lactobacillus crispatus</td>
<td>14.9 (0.5)</td>
<td>97.0 (0.0)</td>
<td>42.5 (0.3)</td>
<td>1.1 (0.0)</td>
<td>26.8 (0.0)</td>
<td>30.1 (12.8)</td>
<td>12.5 (14.1)</td>
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<tr>
<td>Atopobium vaginae</td>
<td>27.7 (0.3)</td>
<td>0.9 (0.0)</td>
<td>31.0 (0.5)</td>
<td>2.5 (3.9)</td>
<td>0.8 (11.5)</td>
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<tr>
<td>Megasphaera elsdenii</td>
<td>1.9 (0.1)</td>
<td>10.5 (2.7)</td>
<td>12.3 (12.3)</td>
<td>0.7 (3.0)</td>
<td>1.1 (5.3)</td>
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<tr>
<td>Staphylococcus epidermidis</td>
<td>34.7 (0.0)</td>
<td>12.4 (5.3)</td>
<td>7.2 (0.5)</td>
<td>2.5 (0.8)</td>
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<td>Enterococcus faecalis</td>
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<td></td>
<td>0.7 (5.0)</td>
<td>2.5 (0.5)</td>
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<td>Ideonella spp.</td>
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<td>0.7 (2.8)</td>
<td>2.5 (2.1)</td>
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<td>Thauera aromatic</td>
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<td>1.3 (4.5)</td>
<td>0.8 (0.0)</td>
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<td>Brevibacillus levickii</td>
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<td>1.7 (3.4)</td>
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<tr>
<td>‘Leptotrichia amnionii’</td>
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<td>1.1 (0.8)</td>
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<td>Peptoniphilus lacrimalis</td>
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<td></td>
<td>0.7 (8.9)</td>
<td>0.8 (8.6)</td>
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<td>Peptoniphilus harei</td>
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<td>Finegoldia magna</td>
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<td>Anaerococcus octavius</td>
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<tr>
<td>Veillonella parvula</td>
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<tr>
<td>Peptoniphilus lacrimalis</td>
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</table>

*Named species with the most similar 16S rRNA sequence from RDP II (Cole et al., 2003) listed in order of prevalence among all samples.

RESULTS AND DISCUSSION

To characterize the microbial diversity found in the normal human vulva, scrape samples from the labia majora and labia minora of four healthy women were analysed. Partial, single-stranded 16S rRNA gene sequences of nearly 900 clones were determined (GenBank accession nos DQ975475–DQ976361) and compared with reference sequence data from RDP II. Each sequence was classified based on the similarity of about 400 nt at the 5’ end of the gene to the corresponding sequence from known bacterial species. The frequencies of phylotypes present in each vulvar sample and their median genetic distance from the most similar 16S rRNA sequence in RDP II are listed in Tables 2 and 3.

The microbiota of the human vulva is diverse

To characterize the microbial diversity found in the normal human vulva, scrape samples from the labia majora and labia minora of four healthy women were analysed. Partial,
use of selective media by previous investigators that allowed them to recover rare populations from their samples. In addition, the sequences of 12 phylotypes diverged by more than 10% from the closest sequence with a species designation in RDP II, indicating that these twelve phylotypes may belong to previously uncharacterized genera or families of bacteria. Eight phylotypes had 5–10% sequence divergence from database sequences and may represent new species within known genera.

A total of 64 phylotypes was recovered from samples of the four women, with diverse populations found. The number of microbial populations varied from two phylotypes in the labia minora of W3 to 27 phylotypes from the labia majora of the same woman. Much of the diversity among sites and women was due to the presence of phylotypes that were unique to each individual (Table 3). Most of the unique phylotypes were not abundant; however, some were found in appreciable numbers (5–10%; Table 3). It should be noted that only numerically abundant populations (those that constituted more than ~1% of the total community) were represented by the sequences reported, and the possible occurrence of less abundant, yet ecologically important, populations cannot be excluded.

**Women have distinctive microbiota**

There were pronounced differences in the composition of vulvar communities of the four women. No two women had the same microbiota and no single phylotype was found in all women. Whilst several phylotypes were detected in more than one woman (Table 2), each woman also had unique phylotypes (Table 3). The most abundant phylotypes in the vulvas of three of the four women were most similar to either *Lactobacillus crispatus* or *Lactobacillus iners*, whilst the community of the fourth (W5) was dominated almost exclusively by phylotypes similar to *L. iners*, *Atopobium vaginae* and a phylotype most similar to *Porphyromonas canis*, *Rhodobacter* spp. (0.9, 5.7%), *Sphingomonas aerolata* (0.9, 1.5%), *Sphingomonas urincola* (1.8, 3.5%), *Sphingomonas yanoikuyae* (8.8, 1.7%), *Staphylococcus haemolyticus* (0.9, 0.0%), *Streptococcus oralis* (0.9, 0.3%) (Table 3).

**Table 3. Phylotypes unique to one location in each woman**

Sample designations (W2–W5) coincide with those of Coolen et al. (2005) and Zhou et al. (2004). A, labia minora; B, labia majora; n, number of clones analysed.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Location</th>
<th>Bacterial species (frequency of phylotype, percentage divergence)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>W2</td>
<td>A (n=105)</td>
<td><em>Aquabacterium citratophilum</em> (1.0, 1.9%), <em>Brevibacillus formosus</em> (1.9, 2.5%), <em>Eubacterium tarantellae</em> (3.8, 8.2%), <em>Paenibacillus lentimorbus</em> (8.6, 14.0%)</td>
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<td></td>
<td>B (n=101)</td>
<td><em>Actinomyces neuii</em> (3.0, 0.5%), <em>Anaerococcus hydrogenalis</em> (1.0, 1.0%), <em>Corynebacterium glucuronolyticum</em> (2.0, 4.1%), <em>Corynebacterium</em> spp. (5.0, 0.5%), <em>Streptococcus anginosus</em> (4.0, 0.1%)</td>
</tr>
<tr>
<td>W3</td>
<td>A (n=101)</td>
<td><em>Lactobacillus jensenii</em> (3.0, 0.5%)</td>
</tr>
<tr>
<td></td>
<td>B (n=114)</td>
<td><em>Acinetobacter johnsonii</em> (1.8, 6.6%), <em>Bacillus jeotgali</em> (1.8, 2.8%), <em>Bacillus psychosaccharolyticus</em> (0.9, 5.3%), <em>Brevundimonas variabilis</em> (1.8, 1.7%), <em>Delftia tsuruhatensis</em> (0.9, 0.0%), <em>Eubacterium desmolans</em> (0.9, 10.6%), <em>Lactococcus lactis</em> (0.9, 0.0%), <em>Legionella pneumophila</em> (0.9, 6.6%), <em>Methylobacterium extorquens</em> (0.9, 1.5%), <em>Methylococcus albus</em> (1.8, 10.5%), <em>Oscillatoriabacter cyanobacterium</em> (0.9, 19.7%), <em>Porphyromonas canis</em> (0.9, 25.3%), <em>Propionibacterium acnes</em> (0.9, 1.5%), <em>Ramlibacter tataouinensis</em> (0.9, 3.2%), <em>Rhodobacter</em> spp. (0.9, 5.7%), <em>Sphingomonas aerolata</em> (0.9, 1.5%), <em>Sphingomonas urincola</em> (1.8, 3.5%), <em>Sphingomonas yanoikuyae</em> (8.8, 1.7%), <em>Staphylococcus haemolyticus</em> (0.9, 0.0%), <em>Streptococcus mitis</em> (2.6, 0.3%), <em>Streptococcus oralis</em> (0.9, 0.3%)</td>
</tr>
<tr>
<td>W4</td>
<td>A (n=106)</td>
<td><em>Paenibacillus validus</em> (1.9, 14.4%)</td>
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<tr>
<td></td>
<td>B (n=87)</td>
<td><em>Bacillus macroides</em> (4.6, 5.3%), <em>Brevibacillus borstelensis</em> (1.1, 8.3%), <em>Staphylococcus hominis</em> (10.3, 0.3%), <em>Staphylococcus lugdunensis</em> (2.3, 0.5%), <em>Staphylococcus piscifermentans</em> (1.1, 0.5%), <em>Thermobacillus xylanilyticus</em> (2.3, 16.7%)</td>
</tr>
<tr>
<td>W5</td>
<td>B (n=120)</td>
<td><em>Anaeroglobus guminatus</em> (3.3, 1.7%), <em>Atopobium minutum</em> (1.7, 9.2%), <em>Dialister pneumosintes</em> (5.8, 12.2%), <em>Eggerthella hongkongensis</em> (0.8, 14.6%), <em>Mobiliuncus curtisi</em> (2.5, 0.0%), <em>Peptoniphilus asacharolyticus</em> (5.0, 15.7%), <em>Peptoniphilus</em> spp. (4.2, 0.9%), <em>Peptostreptococcus ivorii</em> (0.8, 8.1%), <em>Varibaculum cambriense</em> (0.8, 0.5%)</td>
</tr>
</tbody>
</table>

*Named species with the most similar 16S rRNA gene sequence from RDP II (Cole et al., 2003). Data are presented as frequency of each phylotype (i.e. percentage of clones examined from each sample identified as that phylotype). The median genetic divergence (base substitutions per 100 bases) of all sequences of that phylotype from the RDP II sequence of the bacterial species are given as a percentage.

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in the menstrual cycle that the samples were taken (Tables 1–3), although the sample size of this study was too small to make conclusive statements.

The vaginal communities of the women in this study were sampled at the same time that the vulvar samples were taken and their composition has been reported previously (Coolen et al., 2005; Zhou et al., 2004). The dominant phylotypes from the vulva were also dominant members of communities in the corresponding vagina. Indeed, the 16S rRNA gene sequences of the numerically dominant species in each vulvar sample were almost identical to those in the vagina taken from the same subject at the time (Fig. 1; Zhou et al., 2004). Analysis of these vaginal samples using terminal restriction fragment length polymorphisms have indicated that the vaginal community composition is consistent over the menstrual cycle and over a period of at least 2 months (Coolen et al., 2005).

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The vaginal communities of the five women in the study by Zhou et al. (2004) were also commonly encountered among women in a larger study (Forney et al., 2006) that was a subsample of more than 3000 women from across North America (Parsonnet et al., 2005). We expect that a larger study of microbial communities of the vulva will show that vulvar communities are heavily influenced by the vaginal microbiota, and that the communities identified here will reflect healthy vulvar and vaginal communities in general.

Microbiota of the labia majora and labia minora differ and reflect differences in ecotopes

The microbiota of the two regions of the vulva differed from each other, although the dominant phylotypes from the labia minora were generally dominant members of the labia majora communities of the same subject. Communities of the labia majora were more diverse than those of the labia minora, with 2–14 times as many phylotypes.

The community compositions reflected the different ecotopes of these two regions. As noted above, the microbial community of the labia minora resembled that of the vagina. As was found in culture-dependent studies (Aly et al., 1979; Elsner & Maibach, 1990), the microbiota of the labia majora included species found on skin, such as Staphylococcus epidermidis and Corynebacterium spp. In addition, we found populations that were probably of faecal origin, such as Enterococcus faecalis.

Conclusions

These results indicate that the vulva is more complex than originally thought. The small number of participants and single time points suggest that the microbial diversity of the normal human vulva may be even greater than that seen here. It seems likely that various habits and practices of women (such as frequency of bathing, toileting practices and kinds of clothing worn) influence the composition of vulvar communities. Studies that systematically evaluate the effect(s) of such factors are necessary to elucidate the indigenous microbiota of the vulva and identify transient members of these communities.

ACKNOWLEDGEMENTS

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