The Influence of Age and Sex on Ocular Surface Microbiota in Healthy Adults

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Submitted: September 10, 2017
Accepted: October 26, 2017

Increasing evidence indicates that sex hormones influence ocular surface health.1 Immune associated diseases, such as ocular allergy and dry eye, often are caused by alterations in steroid hormone levels.2,3 The aging process also significantly impacts the immune system and eye health, especially with regard to the T cell compartment.4 Moreover, it is well known that the risk of ocular surface diseases increases with age and women are more affected than men,1,4 making it clear that there is a strong relationship among age and sex in immune homeostasis of the ocular surface. Additionally, mounting evidence suggests that diverse commensal microorganisms inhabiting the ocular surface have potent immunoregulatory functions and have an important role in the ocular health and disease pathogenesis.5–8 Although the microbiome is known to be affected by age and sex,9 the influence of these factors on ocular surface microbiota in healthy individuals remains controversial. Using 16S rRNA sequencing, Ozkan et al.10 found no effect of age on the microbial α diversity and a higher Shannon index in males, while a study by Zhou et al.11 indicated no effect of sex on the microbial diversity and a significantly higher richness and Shannon index in children. Moreover, Shin et al.12 reported an effect of sex on the ocular surface microbial community structure.

We characterized the sex- and age-differences in conjunctival microbiome profiles of healthy adults using a metagenomic shotgun sequencing approach. We found that male and female groups differed only in the β diversity of bacterial communities, while there were significant differences in bacterial composition, metabolic functions, and the abundance of antibiotic resistance genes between young and old adult groups.

Conclusions. Our findings suggest that age and sex collectively shape the conjunctival microbiome, and may change the immune homeostasis of the ocular surface through alterations of its commensal microbiome.

Keywords: conjunctival microbiome, metagenomic shotgun sequencing, age and sex
MATERIALS AND METHODS

Subject Recruitment

This study was approved by the institutional review board of Zhongshan Ophthalmic Center, Sun Yat-sen University (protocol #2015MEKY011) and was conducted in accordance with the principles of the Declaration of Helsinki. Informed consent was obtained from all subjects. Nonsmoking healthy volunteers with no signs of systemic disease or ocular disease and no history of antibiotics treatment or contact lens wear in the past 6 months were recruited at Zhongshan Ophthalmic Center. The basic demographic information is listed in the Table.

Sampling of Conjunctival Microbiome

Ocular surface microbiome was sampled by applying a sterile semicircle MF Membrane filter (REF: HAWP01300, 0.45 μm in diameter; Merck Millipore, Burlington, MA, USA) against the inferior bulbar conjunctiva for 10 to 15 seconds. The conjunctival impression cytology samples were placed into sterile Eppendorf tubes, containing 300 μL Tissue and Cell Lysis Solution with 1 μL Proteinase K, provided in the MasterPure Complete DNA and RNA Purification Kit (Epicentre, Amble-side, UK) and immediately frozen at −80°C.

In addition, 11 blank tubes with a membrane that did not contact the conjunctiva were prepared as negative control.

DNA Extraction and Metagenomic Sequencing

DNA was extracted from conjunctival impression cytology samples using the MasterPure Complete DNA and RNA Purification Kit (Epicentre) according to the manufacturer’s instructions. A total of 100 ng DNA was sonicated into fragments of 300 to 400 base pairs (bp) using Bioruptor (Diagenode, Belgium) and then subjected to sequencing library preparation using KAPA LTP Library Preparation Kit (Kapa Biosystems, Wilmington, MA, USA) following manufacture’s protocol. DNA libraries were sequenced on the Illumina HiSeq2500 sequencer using HiSeq PE Cluster Kit v4 and HiSeq SBS V4 250 cycle kit (Illumina, San Diego, CA, USA) and the initial processing was performed by CASAVA (v1.8.2, Illumina).

TABLE. Demographic Characteristics of the Healthy Volunteers and Summary of the Metagenomic Sequencing Data

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th>Old</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of sample (subject)</td>
<td>96 (48)</td>
<td>84 (42)</td>
</tr>
<tr>
<td>Average age</td>
<td>27.9</td>
<td>67.1</td>
</tr>
<tr>
<td>Male/female</td>
<td>23/25</td>
<td>19/23</td>
</tr>
<tr>
<td>Average No. of total reads</td>
<td>51,147,732</td>
<td>33,111,046</td>
</tr>
<tr>
<td>Average No. of nonhuman reads</td>
<td>1,476,867</td>
<td>1,341,236</td>
</tr>
<tr>
<td>Average % of nonhuman reads</td>
<td>2.9</td>
<td>4.1</td>
</tr>
</tbody>
</table>

Figure 1. Relative composition of conjunctival microbiome (A) and positive rate of bacterial species in healthy individuals detected by metagenomic sequencing. (B) Top 20 bacterial species with the most frequent appearance are shown.
FIGURE 2. Relative composition of bacterial community on healthy human conjunctiva. Eleven blank tubes with a membrane that did not contact the conjunctiva were prepared as negative control. The data show almost 100% of the reads are duplications or of low quality.

FIGURE 3. The commensal communities inhabiting the left and right ocular surfaces are statistically indistinguishable. No significant difference is detected in the $\alpha$ diversity (measured by Shannon index, Mann-Whitney $U$ test [A]), $\beta$ diversity (measured by PCA analysis of bacterial relative abundance, $P$ value calculated from a Wilcoxon rank-sum test on samples’ projection onto PC1 [B]), and the relative abundance of core conjunctival bacterial species (Student’s $t$-test, [C]) between the left and right eyes of the same volunteer.
Data Analysis

After quality control by FastQC, the sequence reads were preprocessed with removal of human reads by HiSAT2 (v2.0.1)\(^1\) and DeconSeq\(^1\) to obtain clean nonhuman sequences. The nonhuman sequences were aligned to our custom microbial genome collection using Burrows-Wheeler Aligner (BWA 0.7.5a)\(^1\) and grouped into distinct taxonomic units according to their species level classification. The ratio of the total mapped reads of each species, normalized by the genome size and the total mapped microbial reads within each sample was used to represent the relative abundance of each species. Community diversity (Shannon Index) was calculated according to the method described in the Mothur program.\(^1\)

The abundance of microbial KEGG pathways was analyzed through the use of the HUMAnN2.\(^1\)

Based on the relative abundance of bacterial species and microbial KEGG pathways, principal component analysis (PCA) was performed using Ade4 package in R statistical software (v3.1.1). The nonhuman reads also were mapped to the Antibiotic Resistance Gene Database (ARGDB)\(^1\) using DIAMOND (v0.7.11)\(^1\) to identify the antibiotic resistance genes in each sample. The differences in bacterial species and functional pathways characterizing the groups were evaluated by linear discriminant analysis (LDA) Effect Size (LefSe).\(^2\)

Statistical Analysis

All statistical analyses were implemented in the SPSS software (v17.0). \(P\) values were calculated as indicated in Figure legends using the parametric (Student’s \(t\)-test) or nonparametric (Mann-Whitney \(U\) and Wilcoxon rank-sum) tests. For all boxplots, center lines represent the median and the box edges represent the first and the third quartiles.

RESULTS

Metagenomic Shotgun Sequencing Analysis Reveals Diversity in Ocular Surface Microbiota of Healthy Volunteers

We obtained ocular surface microbiome samples from the inferior bulbar conjunctiva of 48 young (age, 23–44) as well as 42 old (age, 47–84) adults at Zhongshan Ophthalmic Center. A total of 180 samples collected from both eyes of healthy volunteers were subjected to metagenomic shotgun sequencing analysis. Although the majority of all reads were of human origin, an average of approximately 1.4 million nonhuman quality-filtered microbial sequence reads per sample were obtained for subsequent analysis (Table). All 11 blank samples were subjected to the same sequencing library preparation
process and data analysis. The results confirmed that almost 100% of the reads were duplications or of low quality.

Our metagenomic data showed that on average 98.15% of microbial reads were of bacterial origin, while 0.94% and 0.91% were of fungal and viral origins, respectively (Fig. 1A). The ocular surface microbiome was predominated by bacterial species; thus, the following analysis focused on the bacterial community. Propionibacterium acnes could be detected in 88% of healthy volunteers, while the positive rate for Staphylococcus epidermidis was 73% (Fig. 1B). In terms of bacterial community composition, each individual had a unique conjunctival microbiota (Fig. 2). Moreover, we examined whether the bacterial community of the ocular surface differed between the left (OS) and right (OD) eyes of the same volunteer, the results showed that no significant difference was detected in either α (measured by Shannon index, \( P = 0.13 \), Mann-Whitney U test, Fig. 3A) or β (measured by PCA, \( P = 0.15 \), Wilcoxon Rank-Sum test, Fig. 3B) diversity of bacterial composition. Also, there was no significant difference in the relative abundance of core bacterial species (Fig. 3C).

Sex-Differences in Conjunctival Microbiome of Healthy Adults

Next, we investigated the differences in bacterial composition and relative abundance of specific species between male and female groups. No significant difference in α diversity was observed (\( P = 0.094 \), Fig. 4A), but variations in β diversity were significant (\( P < 0.001 \), Fig. 4B). \( P.\) acnes and \( S.\) epidermidis decreased significantly from male to female (\( P < 0.001 \)), while \( E.\) coli increased significantly in the female group (\( P = 0.01 \), Fig. 4C). Core bacterial species are summarized in Figure 4C.

Age and Sex Collectively Shape the Conjunctival Microbiome

We next addressed the question of whether aging affects the ocular surface microbiome. The healthy volunteers were divided into young and old groups. Our results showed that Shannon diversity index score was significantly increased in the old group compared to the young group (\( P = 0.003 \), Fig. 5A). Significant difference also was observed in the β diversity group (\( P < 0.001 \)), and the separation clearly is visible in the PCA plot in Figure 5B. We found a significantly greater abundance of \( S.\) haemolyticus (\( P = 0.042 \)), \( M.\) luteus, and \( E.\) coli in the old compared to the young groups, and a significant decrease in \( O.\) anthropi, \( M.\) hyorhinis, and \( P.\) acnes (\( P < 0.001 \) for all, Fig. 5C) from young to old. The bacterial marker species with significant power to distinguish old and young groups are presented in Figure 6 with linear discriminant analysis scores. Furthermore, when we divided our study groups into young-male, young-female, old-male, old-female groups, the
results showed that the old-female group was the most distinguishable from the other three groups, while no significant difference was identified between young-male and young-female groups (Fig. 7A). Importantly, three antibiotic-resistance genes encoding Class A β-lactamase, BL2b_tem1, and BL2b_tem2, were found highly discriminative only in the microbiome of old adults (Fig. 7B).

Our data also showed significant alterations in metabolic pathways of conjunctival microbiome, especially the carbohydrate and lipid metabolism as well as nucleotide and amino metabolism, between old and young groups (Fig. 8). Taken together, the composition and function of ocular surface microbiome, as well as the abundance of antibiotic resistance genes change with age in healthy adults.

**DISCUSSION**

The human ocular surface is colonized by an expansive, diverse microbial community, as evidenced by previous researches using 16S rRNA sequencing and traditional culture-based methods. The normal microbiota has a protective immunologic role in preventing the proliferation of pathogenic species and alterations in the homeostatic microbiome may be related to ophthalmic pathologies. Thus, it is important to define the influence of age and sex on ocular surface microbiota in healthy adults.

Although the effect of age and sex on the ocular surface microbiome has been investigated by previous studies, the conclusions are controversial: Ozkan et al. found no effect of age on the microbial α diversity and a higher Shannon index in males, while a study by Zhou et al. indicated no effect of sex on the microbial diversity and a significantly higher richness and Shannon index in children younger than 10 years. Moreover, Shin et al. reported an effect of sex on the ocular surface microbial community structure. Our study demonstrated that male and female groups differed only in the β diversity of bacterial communities, while there were
significant differences in bacterial composition and metabolic functions between young and old adult groups, suggesting that age and sex collectively shape the conjunctival microbiome. The inconsistency with previous studies may be explained partly by the use of the metagenomic sequencing approach in our study, which may result in a much broader range of microbes. Interestingly, the conjunctival microbiome of young male and female groups was not distinguishable. However, a significant difference could be found in the composition and function of conjunctival microbiota between old male and female groups, with the most significant difference shown in the old female population. It suggests that during the reshaping of ocular surface microbiome, aging is a stronger factor, while sex only influences microbiome when interacting with age. Previous reports have indicated that a sex-steroid imbalance after menopause initiates profound changes in ocular surface microenvironment and predisposes woman to many autoimmune, inflammatory, and allergic ocular diseases, such as dry eye syndrome.²⁷ It is unclear whether the alteration of conjunctival microbiota found in the old female population is the consequence of sex-hormone imbalance or one of the causes leading to microenvironmental changes on the ocular surface. In addition, several antibiotic resistance genes were enriched in the conjunctival microbiome of old adults, and these genes have been demonstrated to deactivate penicillin and cephalosporin.²⁸ It strongly suggests that the aging process is a risk factor that may change immune homeostasis of the ocular surface through alterations of its commensal microbiome and lead to the acquirement of bacterial strains with antibiotic resistance.

Our study has several limitations. All subjects are living in the south of China. It is conceivable that populations living in different regions or different environments might demonstrate a distinct conjunctival microbiome. Further studies are needed to illustrate how these communities are affected and the contribution of ocular microbiome to disease pathology.

**CONCLUSIONS**

The healthy ocular surface microbiome is shaped collectively by age and sex, and the aging process may be a stronger factor. They may change the immune homeostasis of the ocular surface through alterations of its commensal microbiome.

**Acknowledgments**

The authors thank all members of the Wei Laboratory for their support and discussion.

Supported by the National Basic Research Program of China 2015CB964601 (LW), and by the National Basic Research Program of China 2013CB967001, Li Foundation Heritage Prize, 1000 Young Talent Plan China, National Natural Science Foundation of China and the National Natural Science Foundation of China.
Chen 81570828, and NSFC-Guangdong Fund for Application of Supercomputing using The Tianhe-2 Supercomputer (LW).

Disclosure: X. Wen, None; L. Miao, None; Y. Deng, None; P.W. Bible, None; X. Hu, None; Y. Zou, None; Y. Liu, None; S. Guo, None; J. Liang, None; T. Chen, None; G.-H. Peng, None; W. Chen, None; L. Liang, None; L. Wei, None

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