Alterations in the Ocular Surface Microbiome in Traumatic Corneal Ulcer Patients

Yutong Kang,1 Hao Zhang,1 Meina Hu,1 Yao Ma,1 Pengfei Chen,2 Zelin Zhao,2 Jinyang Li,2 Yuee Ye,2 Meiqin Zheng,1,2 and Yongliang Lou1

1Zhejiang Provincial Key Laboratory for Technology and Application of Model Organisms, Key Laboratory of Laboratory Medicine, Ministry of Education China, School of Laboratory Medicine and Life Sciences, Wenzhou Medical University, Wenzhou, China
2School of Ophthalmology and Optometry and Eye Hospital, School of Biomedical Engineering, Wenzhou Medical University, Wenzhou, China

Correspondence: Meiqin Zheng, Zhejiang Provincial Key Laboratory for Technology and Application of Model Organisms, Key Laboratory of Laboratory Medicine, Ministry of Education, School of Laboratory Medicine and Life Sciences, Wenzhou Medical University, Wenzhou, Zhejiang, China; zmq@eye.ac.cn.

Yongliang Lou, Zhejiang Provincial Key Laboratory for Technology and Application of Model Organisms, Key Laboratory of Laboratory Medicine, Ministry of Education, School of Laboratory Medicine and Life Sciences, Wenzhou Medical University, Wenzhou, Zhejiang, China; louyongliang2013@163.com.

Received: January 16, 2020
Accepted: March 15, 2020
Published: June 16, 2020

Citation: Kang Y, Zhang H, Hu M, et al. Alterations in the ocular surface microbiome in traumatic corneal ulcer patients. Invest Ophthalmo Vis Sci. 2020;61(6):35. https://doi.org/10.1167/iovs.61.6.35

Purpose. Corneal ulcers are a common eye inflammatory disease that can cause visual impairment or even blindness if not treated promptly. Ocular trauma is a major risk factor for corneal ulcers, and corneal trauma in agricultural work can rapidly progress to corneal ulcers. This study aims to evaluate the changes in the ocular surface (OS) microbiome of patients with traumatic corneal ulcer (TCU).

Methods. Among 20 healthy control (HC) subjects and 22 patients with TCU, 42 eyes were examined to investigate the OS microbial flora using metagenomic shotgun sequencing.

Results. At the taxonomic composition level, our findings showed that dysbiosis (alterations in richness and community structure) occurs in the OS microbiome of patients with TCU. Notably, Pseudomonas was present at a greater than 30% relative abundance in all individuals in the TCU group. At the species level, the abundance of Pseudomonas fluorescens and Pseudomonas aeruginosa was significantly elevated in the TCU group compared to the HC group. At the functional level, we identified significant differences in the HC and TCU groups. We observed that inflammation-related pathways involved in bacterial chemotaxis, flagellar assembly, and biofilm formation were significantly more abundant in the TCU group. Besides, the pathways related to biosynthesis, degradation, and metabolism were also increased significantly in the TCU group.

Conclusions. These findings indicate an altered OS microbiome in the affected eyes of patients with TCU. Further research is needed to determine whether these alterations contribute to the pathogenesis of TCU or impact disease progression.

Keywords: traumatic corneal ulcer, ocular surface microbiome, shotgun metagenomics

The microbiota of the OS is an emerging field of research. The characteristics of the eye include an external surface composed of mucosal tissues, including the palpebral conjunctiva, the bulbar conjunctiva, and the fornix conjunctiva.1 The human OS harbors a variety of bacteria, fungi, and viruses due to continuous exposure to the environment.2 The ocular commensal organisms play a key role in defending against pathogens and maintaining immune homeostasis. Nevertheless, once the integrity of the OS is destroyed, protection is lost.3 In the setting of corneal injuries, local environmental changes and contamination may exacerbate the growth and invasion of pathogenic and opportunistically pathogenic organisms. Living microorganisms and their products can activate potential adaptive immune responses and lead to disease.4–6 Various complications will be induced if corneal injury cannot be treated effectively and in a timely manner, such as corneal ulcers, recurrent erosion, and loss of vision.7 In some developing countries, corneal trauma occurring during agricultural work was shown to be the main factor predisposing individuals to corneal ulceration.8,9 Compared with some industrialized countries, the incidence of ocular trauma may be higher in China.10 The purpose of this research was to evaluate changes in the OS microbiome of patients with traumatic corneal ulcer (TCU), providing valuable information for clinical diagnosis and treatment.

Although the composition of the ocular microbiome is still under dispute, data have become available to indicate the distribution characteristics of OS microbial communities in health and disease states. Recent studies have demonstrated the potential relationship between changes in the OS microbiome and some conditions, such as trachoma,11...
fungal keratitis,12,13 ulcerative bacterial keratitis,14 conjunctivitis,15 dry eye,16 mesangial gland dysfunction,17,18 blepharitis,19 and contact lens wearing.20,21 However, all of these activities, dry eye, mesangial gland dysfunction, blepharitis, and contact lens wearing are correlated with TCU.

To characterize the compositional and functional profiles of the OS microbiome, we performed a shotgun metagenomics survey on the OS microbiome of 22 patients with TCU and 20 HC subjects to characterize the compositional and functional changes correlated with TCU.

**Materials and Methods**

**Ethical Permission**

The study protocol was approved by the Ethics Committee of the Eye Hospital of Wenzhou Medical University. This study adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all subjects at the time of sample collection.

**Study Participants**

This study enrolled 22 patients with TCU (age 42–68 years) and 20 HC subjects (age 43–68 years). Participants were enrolled from a combination of patients presenting at the outpatient department at the Eye Hospital of Wenzhou Medical University and middle-aged and elderly persons living in communities across Wenzhou, China. All patients in the TCU group met the criteria for the clinical diagnosis of corneal ulcers: the presence of a corneal epithelial defect with associated supplicative infiltrate, with or without hypopyon, and were accordingly diagnosed with TCU. The agents responsible for corneal trauma were mainly agricultural products (Supplementary Table S1). The healthy volunteers had no history of systemic or ocular diseases or contact lens wear. All samples from the HC and TCU groups were free of topical or systemic antibiotics or steroids from treatment within 6 months.

**Sample Collection and Processing**

Sample collection was performed in an ophthalmic treatment room sterilized by ultraviolet irradiation. Samples were collected from ocular surface mucosal tissues (upper and lower palpebral, caruncle, and conjunctival fornix) using flocced swabs of the Copan ESWab transport system (Copan Diagnostics Inc., Murrieta, CA, USA) after the instillation of sterile topical proparacaine. All patients with TCU developed unilateral eye disease. A randomly chosen eye from each HC subject was sampled as a control. To avoid contamination, another environmental “air swab” containing the topical anesthetic was prepared as a negative control. Collected swabs were immediately placed on ice and transferred to the laboratory to be frozen at -80°C until sequencing. No DNA was detected in the “air swabs” using a Qubit 2.0 Fluorometer, and no DNA bands were found by performing 1% agarose gel electrophoresis on the amplified product of the 16S rRNA gene V3 to V4 region (30 cycles).

**Metagenomic Shotgun Sequencing**

Genomic DNA was sequenced on an Illumina HiSeq X10 platform (Novogene Co., Ltd., Beijing, China) using the metagenomic shotgun sequencing method (2 × 150 cycle runs). After quality inspection by FastQC, the adapter was trimmed by Cutadapt, and low-quality reads were filtered out using Trim Galore.25 High-quality readings were visualized by the tool SplicingViewer.24 Trimmed reads were mapped to the human reference genome (hg19) using Bowtie2 (version 2.3.4.3).25 Using SamTools (version: 0.1.19), aligned reads were removed to obtain clean nonhuman sequences.26 Then, the remaining reads were assembled by Megahit (version 1.1.3),27 and the contigs were submitted to MetaGeneMark (version 3.38) for prediction.28 Redundant amino acid sequences were excluded using CD-HIT (version 4.6) and a threshold of ≥90% sequence identity.29 We mapped the nonredundant amino acid catalogs to an integrated National Register (NR) database (including bacteria, archaeabacteria, fungi, and viruses) with Diamond (version 0.8.22.84).30 The hit results were submitted to Megan (version 6) with the weighted lowest common ancestor (LCA) algorithm to assess the taxonomic compositions.31 Functional analysis was carried out via DIAMOND search against the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Clusters of Orthologous Group (COG) databases.

**Statistical Analysis**

Statistical analysis was performed using R 3.6.0. Rarefaction curves of the microbiomes were plotted using the Vegan package in R. Alpha diversity was evaluated by the Shannon index and Simpson index. A t-test was used to test the differences in the Shannon diversity and Simpson index between the two groups. Principal coordinate analysis (PCOA) with the Bray-Curtis dissimilarities and Jaccard index was used to explore microbial community structure. Permutational multivariate ANOVA (PERMANOVA) was performed for beta diversity analysis. The Kruskal–Wallis test was applied to identify the phylotypes and functional pathways with significantly different relative abundances between HC subjects and patients with TCU; phylotypes and functional pathways with a Q < 0.01 were regarded as significantly enriched. To identify environment-associated biomarker taxa, linear discriminant analysis (LDA) effect size (LeSe) was used. An LDA score > 5 was considered to indicate a significant biomarker. The R package “RandomForest” was used to obtain the taxonomic contributions of microbial communities at the species level. The SparCC algorithm was used to obtain the correlations and P values between genus abundances (from at least three subjects) in each group.32 The interaction networks were visualized with Cytoscape3.6.0.33
RESULTS

Metagenomic Data Analysis

A total of 42 subjects were included with matched age (Mann–Whitney U test, \( P = 0.97 \)) and sex (Fisher’s exact test, \( P = 1.0 \)) between the TCU (\( n = 22 \)) and HC (\( n = 20 \)) groups (Table, Supplementary Table S1). In total, > 5.19 billion reads were obtained, and an average of 3.38 million reads per sample were used for further analysis (Table). After trimming and filtering, the low percentage of the remaining reads might have been due to the dominance of human genomes, as human saliva, nasal cavity, skin, and vaginal specimens routinely have > 90% human content.34

Alpha Diversity and Beta Diversity of the Ocular Microbiome

Rarefaction analysis showed that the species richness in each group approached saturation, implying that the current sequencing depth covered the largest species diversity (Supplementary Fig. S1). The Shannon’s diversity index and Simpson’s diversity index based on the genus profiles in the TCU group were significantly lower than those in the HC subjects (Kruskal–Wallis test; \( P = 0.0059 \) for Shannon index and \( P = 0.0035 \) for Simpson index; Fig. 1C, D). The alpha diversity results indicated that the richness and evenness of the OS microbial communities in the TCU group were also significantly lower. PCOA and PERMANOVA analysis showed that the OS microbiome community structure of patients with TCU was significantly different from that of controls based on Bray–Curtis dissimilarities and the Jaccard index (\( P = 0.007992 \) for Bray–Curtis dissimilarities and \( P = 0.001998 \) for Jaccard index; Fig. 1E, F).

Taxonomic Changes in the OS Microbiome

Overall, the detected taxa compositions included four microbial kingdoms, including bacteria, fungi, archaea, and viruses (Supplementary Figure S2). Bacteria were the most abundant kingdom, representing > 90% of the relative abundance in each individual. Bacteria and fungi were shared by all samples, yet archaea and viruses were not found on all OSs. No archaea were found in patients with TCU. Compared to the HC group, the mean relative abundance of bacteria was higher in the TCU group, whereas the mean relative abundance of fungi was lower (Fig. 1A, B).

All kingdoms were classified into 19 phyla and 163 genera. At the phylum level, the OS microbiome was dominated by three phyla: Proteobacteria (TCU 75.61%; HC 28.35%), Actinobacteria (TCU 2.37%; HC 29.61%), and Firmicutes (TCU 13.0%; HC 35.72%). Other phyla with > 1% average relative abundance in each group included Bacteroidetes (TCU 3.05%; HC 1.35%), Chlamydiae (TCU 0.58%; HC 1.23%), Deinococcus-Thermus (TCU 3.55%; HC 0.15%), and Mucoromycota (TCU 0.19%; HC 1.0%) (Fig. 1G). The LEfSe analysis identified three biomarkers at the phylum level. Actinobacteria and Firmicutes were potential biomarkers for the HC group, whereas Proteobacteria was overrepresented in the TCU group (Fig. 1H). Compared with the HC group, the TCU group contained significantly lower levels of Actinobacteria, Firmicutes, Mucoromycota, and Chlamydiae and markedly higher levels of Proteobacteria (Fig. 1I).

As shown in Figure 2A, 15 genera with a mean relative abundance > 1% were found, including Pseudomonas, Streptococcus, Corynebacterium, Cronobacter, Staphylococcus, Escherichia, Meiothermus, Vibrio, Mycobacterium, Chlamydia, Clostridioides, Mycobacteroides, Paenibacillus, Pseudoalteromonas, and Alistipes. Of these, the top five most abundant genera were Pseudomonas (TCU 57.49%; HC 0.47%), Streptococcus (TCU 4.24%; HC 22.93%), Corynebacterium (TCU 0.53%; HC 19.42%), Cronobacter (TCU 0.83%; HC 80.03%), and Staphylococcus (TCU 2.25%; HC 3.83%). LEfSe analysis identified Pseudomonas and Corynebacterium as biomarkers for the TCU and HC groups, respectively (Fig. 2B). Notably, Pseudomonas was present at a relative abundance of > 30% in all patients with TCU. Interestingly, subjects HC5, HC7, HC16, and HC18 showed high dominance by Corynebacterium, accounting for 75.36%, 78.48%, 72.05%, and 53.95%, respectively (Fig. 2C). Our previous research has demonstrated that some Corynebacterium spp. could produce amino acids in large quantities.35 Twelve genera were found to be differentially abundant between the groups. Pseudomonas, Meiothermus, and Alistipes were significantly enriched in the TCU group, and Chlamydia, Clostridioides, Corynebacterium, Cronobacter, Mycobacterium, Mycobacteroides, Paenibacillus, Staphylococcus, and Streptococcus were enriched in the HC group (Fig. 2D).

At the species level, the OS microbiome in the HC and TCU groups was categorized into 274 species, with 36.29 ± 9.37 (range, 18-64) species detected in the samples from each individual. Of note, unclassified species accounted for a high proportion (range, 13.26%-86.42%). Although some individuals showed a dominance of Corynebacterium and Pseudomonas at the genus level, unclassified Pseudomonas species and Corynebacterium species accounted for the majority. Figure 3A shows the top 20 species in the TCU and HC groups. Among these species, 14 were found to differ significantly between the HC group and the TCU group. Compared with the HC subjects, the OS microbiome of the TCU group had higher abundances of Pseudomonas aeruginosa, Pseudomonas fluorescens, and Meiothermus silvanus and lower abundances of Rhizobius irregularis, Streptococcus pneumoniae, Mycobacteroides abscessus, Clostridioides difficile, Strepto
coccus pyogenes, Corynebacterium accolens, Paenibacillus odorifer, Cronobacter sakazakii, Mycobacterium tuberculosis, Thalassospira xiamenensis, and Pseudoalteromonas luteoviolacea (Fig. 3B). Because LEfSe analysis found no biomarker taxa in the species, the random forest algorithm was selected as an alternative selection method to determine the variable importance. The MeanDecreaseAccuracy and MeanDecreaseGini values of all different species were > 0.1. Among these, Pseudomonas fluorescens and Pseudomonas aeruginosa had the strongest classification contributions (Fig. 3C, D). Pseudomonas fluorescens was found in all patients with TCU. Except for subject TCU3, Pseudomonas aeruginosa was present in the OS microbial community of all patients with TCU (Supplementary Fig. S3).
In the HC group, only three viruses, namely, *Torque teno virus* (TTV), *Gammagapillomavirus 8*, and *Ateline gammaherpesvirus 3*, were detected. In the study by Doan et al., TTV was found on 65% of the OSs of HC subjects. Previous work found that herpes virus was present in the tears of HC subjects. Although the richness of the OS microbiome of the TCU group was decreased, the number of virus types in the TCU group was obviously increased compared to that in the HC group. The viruses included *Betapapillomavirus 1*, *Betapapillomavirus 2*, *Betapapillomavirus 3*, *Eel River basin pequenovirus*, *Human alphaherpesvirus 1*, *Human polyomavirus 5*, *Microviridae Fen7918_21*, and TTV (Supplementary Fig. S4). The increased number of virus types may be related to the introduction of contamination in cases of...
eye injury and to the reduced ability to remove pathogens after OS homeostasis has developed an imbalance.

**Correlation and Co-Occurrence Analyses of the OS Microbiome**

To investigate the co-occurrence of OS microorganisms, we constructed interaction networks based on pairwise correlations between the relative abundances of the different genera (Fig. 4A, B). Overall, the strength of the microbial co-occurrence for the TCU group was weaker than that for the HC group, suggesting possible ecological disturbance of the ocular microbiome in patients with corneal ulcers. In the HC group, 10 genera (with > 10 interactions), namely, *Clostridoides*, *Cronobacter*, *Escherichia*, *Mycobacterium*, *Streptococcus*, *Vibrio*, and *Thalassospira*, were identified, indicat-
FIGURE 4. Microbial correlation based on relative abundance. Interaction network in the OS microbiome of HC subjects (A) and patients with TCU (B) (Spearman correlation magnitude > 0.4 and q < 0.05 are shown). Each node represents a genus (relative abundance ≥ 0.5% in at least one group), and the size of the nodes is proportional to their degree of interaction. The co-abundance (positive correlation) and co-exclusion (negative correlation) are indicated by green and red connections, respectively.

ing their possible key roles in the network. In the TCU group, no genera had connectivity > 10. Interestingly, in the interaction network of the HC group, only Corynebacterium was negatively related to other genera, and Corynebacterium exhibited only negative interactions.

Functional Alterations in the OS Microbiome
At present, the functional profiles of the OS microbiome are still poorly understood. In this study, we investigated the differences in functional pathways between the HC group and TCU group based on a substantial amount of metagenomic shotgun sequencing data. The metagenomic genes of all samples were mapped onto KEGG orthologous groups and the COG database. The overall functional profiles were significantly different between the HC group and the TCU group (Fig. 5A, B; Supplementary Fig. S5B, C). Among bacterial pathways using the KEGG orthologous group annotation, a total of 53 differential pathways were found, of which 52 were significantly enriched in the TCU group, and only the synthesis of tyrosine was enriched in the HC group. Genes related to metabolism, degradation, and biosynthesis were significantly increased in the TCU group. All subjects had a higher abundance of genes related to ABC transporters, the two-component system, glyoxylate and dicarboxylate metabolism, fatty acid metabolism, fatty acid degradation, and folate biosynthesis.
FIGURE 5. KEGG functional pathways of the OS microbiome. PCOA plots of Bray–Curtis dissimilarities (A) and Jaccard index (B) in which samples were colored based on grouping. (C) The relative abundances of 53 KEGG functional pathways were significantly different in the TCU group and in the HC group.

A total of 23 functions of COG categories were annotated. Among them, seven COG categories related to metabolism were found, including amino acid transport and metabolism, carbohydrate transport and metabolism, coenzyme transport and metabolism, inorganic ion transport and metabolism, lipid transport and metabolism, nucleotide transport and metabolism, and secondary metabolite biosynthesis, transport, and catabolism. Whether the metabolic activity of the OS microbiome plays a role in maintaining OS homeostasis is worthy of further research. Compared with the HC group, the genes related to secondary metabolite biosynthesis, transport, and catabolism, cell wall/membrane/envelope biogenesis, energy production and conversion, lipid transport and metabolism, and inorganic ion transport and metabolism were significantly overrepresented in patients with TCU, whereas cell cycle control, cell division, chromo-
Discrimination of fungus can protect eyes against Candida albicans and Pseudomonas aeruginosa infections by facilitating neutrophil recruitment. Our research did not find Corynebacterium mastitidis and whether other Coryneform species have similar effects remains to be further studied.

At the species level, the composition and relative abundance varied markedly among individuals, possibly depending on physiological differences, environment, and lifestyle. Among the top 20 species in the HC group, Chlamydia trachomatis, Staphylococcus aureus, Escherichia coli, and Streptococcus pneumoniae are well-known ocular pathogens, which implies that a healthy OS has powerful mechanisms to suppress pathogens from exerting pathogenic effects.37 OS infection results from virulence being enhanced by external factors, such as antibiotics, infection, preservatives, surgery, the insertion of removable contact lenses, and other surface disorders.

In this study, we demonstrated changes in the OS microbiome functional spectrum in patients with TCU. All patients with TCU had higher abundances of fatty acid degradation- and metabolism-related genes than the HC individuals. Among the COG categories that were identified, the genes related to lipid transport and metabolism were also obviously increased in the TCU group. Lipids are an important component of the tear film, which can prevent OS dewetting and water evaporation and provide a smooth refractive layer.38,39 Lipid-based products are effective in the treatment of dry eye.40 To determine whether OS microbial communities can synthesize lipids and transport them to the OS to participate in OS lubrication, animal-based research is needed.

Using the KEGG orthologous group annotation, we observed that the genes related to flagellar assembly and bacterial chemotaxis were more abundant in the TCU group than the HC group. Bacteria can move toward favorable conditions and away from adverse environments through chemotaxis-guided movements, which play an important role in the onset of post-traumatic corneal infections. Interestingly, the results of alpha diversity analysis indicated that the richness of the OS microbial community in the TCU group was decreased, but the genes related to metabolism, degradation, and biosynthesis were significantly increased at the functional level. The reason may be that OS trauma may destroy the innate immune system of the corneal epithelium, resulting in uncontrolled growth and metabolism of the OS flora.

Notably, genes related to biofilm formation by Pseudomonas aeruginosa and Escherichia coli were overrepresented in the TCU group. Bacterial biofilm was defined as “sessile bacterial communities growing on a surface.” Compared with free-living or planktonic bacteria, bacteria in biofilms are more resistant to antibiotics and to the host immune response.41 Because the host immune response and antimicrobial therapies have difficulty eliminating bacteria growing in biofilms, a chronic inflammatory response may be produced at the site of the biofilm.42 Earlier studies have shown that P. aeruginosa cannot colonize healthy corneal epithelial cells well, but its adherence is significantly increased when the corneal epithelium is damaged.43

In addition, we also found that genes related to virus carcinogenesis were enriched in the TCU group. Compared to the HC group, TCU group had clearly increased numbers of virus types. Possible associations between eye neoplasms and viruses include hepatitis C in ocular adnexal MALT.
lymphoma, herpes virus 8 in Kaposi sarcoma, human immunodeficiency virus in conjunctival squamous cell carcinoma, and human papillomavirus in conjunctival papilloma and squamous cell carcinoma. Further research is needed on whether the imbalance of OS homeostasis increases the risk of virus carcinogenesis.

There are several limitations in our study. First, although our results are statistically supported, the sample size is insufficient. In China, because most eye medicines are sold as nonprescription drugs at a pharmacy, the majority of patients with ocular injuries began to use topical medication before the initial visit to the Eye Hospital of Wenzhou Medical University. Therefore, we recruited only 22 patients with TCU without any history of medication use from February 2018 to September 2019. Our team is also enrolling more subjects, and further studies will compare patients with keratitis with or without corneal ulcers to explore the association between OS microbiome changes and disease severity. Second, most of our subjects are middle-aged and elderly persons, which may lead to a certain age bias in the study results.

CONCLUSIONS

Overall, we have clearly described the taxonomic composition, functional profiles and microbial co-occurrence of the OS microbiome in patients with TCU and HC subjects. The results of shotgun metagenomics analysis will provide important references for clinical diagnosis and treatment. The results are also of great importance for the future development of probiotic eye drops for treating corneal ulcers.

Acknowledgments

The authors thank Jinyu Wu of the Institute of Genomic Medicine, Wenzhou Medical University, for his support and help with data analysis. The authors also thank the Key Discipline of Zhejiang Province in Medical Technology (First Class, Category A).

Supported by the Science and Technology Bureau of Wenzhou (Y20190171).

Disclosure: Y. Kang, None; H. Zhang, None; M. Hu, None; Y. Ma, None; P. Chen, None; Z. Zhao, None; J. Li, None; Y. Ye, None; M. Zheng, None; Y. Lou, None

References

1. Doan T, Akileswaran L, Andersen D, et al. Paucibacterial microbiome and resident DNA virome of the healthy conjunctiva. Invest Ophthalmol Vis Sci. 2016;57:5116–5126.
2. Wen X, Miao L, Deng Y, et al. The influence of age and sex on ocular surface microbiota in healthy adults. Invest Ophthalmol Vis Sci. 2017;58:6050–6057.
3. Miller D, Iovieno A. The role of microbial flora on the ocular surface. Curr Opin Allergy Clin Immunol. 2009;9:466–470.
4. Edelman SM, Kasper DL. Symbiotic commensal bacteria direct maturation of the host immune system. Curr Opin Gastroenterol. 2008;24:720–724.
5. Gilger BC. Immunology of the ocular surface. Vet Clin North Am Small Anim Pract. 2008;38:223–231, v.
6. Rakof-Nahoum S, Medzhitov R. Role of the innate immune system and host-commensal mutualism. Curr Top Microbiol Immunol. 2006;308:1–18.
7. Ahmed F, House RJ, Feldman BH. Corneal abrasions and corneal foreign bodies. Prim Care. 2015;42:363–375.
8. Khanal B, Deb M, Panda A, et al. Laboratory diagnosis in ulcerative keratitis. Ophthalmic Res. 2005;37:125–127.
9. Srinivasan M. Prevention of traumatic corneal ulcer in South East Asia. Community Eye Health. 2017;30:S15–S17.
10. Wang W, Zhou Y, Zeng J, et al. Epidemiology and clinical characteristics of patients hospitalized for ocular trauma in South-Central China. Acta Ophthalmol. 2017;95:e503–e510.
11. Zhou Y, Holland MJ, Makalo P, et al. The conjunctival microbiome in health and trachomatous disease: a case control study. Genome Med. 2014;6:99.
12. Prashanthi GS, Jayasudha R, Chakravarthy SK, et al. Alterations in the ocular surface fungal microbiome in fungal keratitis patients. Microorganisms. 2019;7:309.
13. Ge C, Wei C, Yang BX, et al. Conjunctival microbiome changes associated with fungal keratitis: metagenomic analysis. Int J Ophthalmol. 2019;12:194–200.
14. (Tuzhikov A, et al. IOVS 2013;54:ARVO E-Abstract 2891).
15. Yau JW, Hou J, Tsai SKW, et al. Characterization of ocular and nasopharyngeal microbiome in allergic rhinoconjunctivitis. Pediatr Allergy Immunol. 2019;30:624–631.
16. Graham JE, Moore JE, Jiru X, et al. Ocular pathogen or commensal: a PCR-based study of surface bacterial flora in normal and dry eyes. Invest Ophthalml Vis Sci. 2007;48:5616–5623.
17. Jiang X, Deng A, Yang J, et al. Pathogens in the meibomian gland and conjunctival sac: microbiome of normal subjects and patients with meibomian gland dysfunction. Infect Drug Resist. 2018;11:1729–1740.
18. Dong X, Wang Y, Wang W, et al. Composition and diversity of bacterial community on the ocular surface of patients with meibomian gland dysfunction. Invest Ophthalml Vis Sci. 2019;60:4774–4783.
19. Lee SH, Oh DH, Jung JY, et al. Comparative ocular microbial communities in humans with and without blepharitis. Invest Ophthalml Vis Sci. 2012;53:5585–5593.
20. Shin H, Price K, Albert L, et al. Changes in the eye microbiota associated with contact lens wearing. MBio. 2016;7:e01998.
21. Zhang H, Zhao F, Hutchinson DS, et al. Conjunctival microbiome changes associated with soft contact lens and orthokeratology lens wearing. Invest Ophthalml Vis Sci. 2017;58:128–136.
22. Quince C, Walker AW, Simpson JT, et al. Corrigendum: shotgun metagenomics, from sampling to analysis. Nat Biotechnol. 2017;35:833–844.
23. Wang T, Liu Q, Li X, et al. RRBS-analyser: a comprehensive web server for reduced representation bisulfite sequencing data analysis. Hum Mutat. 2013;34:1606–1610.
24. Liu Q, Chen C, Shen E, et al. Detection, annotation, and visualization of alternative splicing from RNA-Seq data with SplicingViewer. Genomics. 2012;99:178–182.
25. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods. 2012;9:357–359.
26. Li H, Handsaker B, Wysoker A, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics. 2009;25:2078–2079.
27. Li D, Liu CM, Luo R, et al. MEGAHT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. Bioinformatics. 2015;31:1674–1676.
28. Zhu W, Lomsadze A, Borodovsky M. Ab initio gene identification in metagenomic sequences. Nucleic Acids Res. 2010;38:e132.
29. Fu L, Niu B, Zhu Z, et al. CD-HIT: accelerated for clustering the next-generation sequencing data. *Bioinformatics*. 2012;28:3150–3152.
30. Buchfink B, Xie C, Huson DH. Fast and sensitive protein alignment using DIAMOND. *Nat Methods*. 2015;12:59–60.
31. Huson DH, Beier S, Flade I, et al. MEGAN Community Edition - Interactive exploration and analysis of large-scale microbiome sequencing data. *PLoS Comput Biol*. 2016;12:e1004957.
32. Friedman J, Alm EJ. Inferring correlation networks from genomic survey data. *PLoS Comput Biol*. 2012;8:e1002687.
33. Shannon P, Markiel A, Ozier O, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res*. 2003;13:2498–2504.
34. Marotz CA, Sanders JG, Zuniga C, et al. Improving saliva shotgun metagenomics by chemical host DNA depletion. *Microbiome*. 2018;6:42.
35. Liu G, Wu J, Yang H, et al. Codon usage patterns in *Corynebacterium glutamicum*: mutational bias, natural selection and amino acid conservation. *Comp Funct Genomics*. 2010;2010:343–569.
36. Kaufman HE, Azcuy AM, Varnell ED, et al. HSV-1 DNA in tears and saliva of normal adults. *Invest Ophthalmol Vis Sci*. 2005;46:241–247.
37. Dong Q, Brulc JM, Iovieno A, et al. Diversity of bacteria at healthy human conjunctiva. *Invest Ophthalmol Vis Sci*. 2011;52:5408–5413.
38. Huang Y, Yang B, Li W. Defining the normal core microbiome of conjunctival microbial communities. *Clin Microbiol Infect*. 2016;22:643e7–643e12.
39. Ozkan J, Nielsen S, Diez-Vives C, et al. Temporal stability and composition of the ocular surface microbiome. *Sci Rep*. 2017;7:9880.
40. St Leger AJ, Desai JV, Drummond RA, et al. An ocular commensal protects against corneal infection by driving an interleukin-17 response from mucosal gammadelta T cells. *Immunity*. 2017;47:148–158, e5.
41. Bron AJ, Tiffany JM, Gouveia SM, et al. Functional aspects of the tear film lipid layer. *Exp Eye Res*. 2004;78:347–360.
42. Pucker AD, Haworth KM. The presence and significance of polar meibum and tear lipids. *Ocul Surf*. 2015;13:26–42.
43. Garrigue JS, Amrane M, Faure MO, et al. Relevance of lipid-based products in the management of dry eye disease. *J Ocul Pharmacol Ther*. 2017;33:647–661.
44. Zegans ME, Shanks RM, O’Toole GA. Bacterial biofilms and ocular infections. *Ocul Surf*. 2005;3:73–80.
45. Zegans ME, Becker HI, Budzik J, et al. The role of bacterial biofilms in ocular infections. *DNA Cell Biol*. 2002;21:415–420.
46. Spurr-Michaud SJ, Barza M, Gipson IK. An organ culture system for study of adherence of *Pseudomonas aeruginosa* to normal and wounded corneas. *Invest Ophthalmol Vis Sci*. 1988;29:379–386.