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Lacrimal Apparatus, Eyelids, Orbit

Validation of 16S rRNA Gene Sequencing of the Periocular Microbiome and Lack of Alteration by Topical Eyedrops

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Purpose: Genomic techniques for characterizing the ocular microbiome require further validation. We compared the microbiome of patients' eyelids through both conventional culture and 16S rRNA analysis and analyzed the impact of eyedrop use on microbiome diversity.

Methods: Ninety-eight patients followed for management of glaucoma or suspicion of glaucoma had eyelid swabs performed with Isohelix MS Mini DNA Swabs (98 participants) and ESwabs (49 participants) for 16S rRNA analysis and conventional culture, respectively. The effect of preservative-containing eyedrops on the microbiomes detected using these two techniques were analyzed and compared across techniques.

Results: Forty-five of the 50 (non-unique) genera (90%) identified by conventional culture were also identified by each individual's 16S rRNA analysis within the top 14 most abundant organisms present based on operational taxonomic unit. All conventional cultures performed had at least one or more genera also identified by each participant's 16S rRNA analysis. There was no difference in the conventional culture positivity rate or proportion of participants with a particular genus present on conventional culture based on whether preservative-containing eyedrops were regularly used. Similarly, in eyes using versus not using eyedrops, no differences were observed in the proportions of participants with a particular genus present or the Shannon index as determined by 16S rRNA analysis.

Conclusions: 16S rRNA analysis correlates well with conventional culture results for the eyelid microbiome, with results from neither technique demonstrating an association of microbiome composition and eyedrop use. The clinical relevance of the large numbers of microbes detected via 16S rRNA analysis requires further study.

Translational Relevance: 16S rRNA analysis of the periocular microbiome is consistent with conventional culture and enables further study of physiologic and pathologic ocular processes possibly related to microbiome diversity.

Introduction

Characterization of the human microbiome is an active area of research as its importance continues to be elucidated in both the eye[1–5] and the body as a whole.[6,7] The role of the ocular microbiome may be relevant to the diverse pathology of both anterior and posterior segments.[3] Despite the potential importance of the ocular microbiome, methods for its characterization still require optimization, and little is known about how chronic eyedrop use may potentiate changes in its composition and alter the presence and/or course of ocular disease. Eyedrops used to treat chronic conditions such as glaucoma often have preservatives such as benzalkonium chloride to prevent bacterial growth within the solution. However, preservatives also alter the normal microbial composition of the ocular surface and surrounding structures, and the clinical

importance of these changes is poorly understood.[8–11] Alteration of the native ocular microbiome may predispose patients to ocular discomfort, surface inflammation and scarring, or colonization with different, and possibly more virulent, organisms capable of causing infection.[1,9–11]

Evaluation of the microbiome has increasingly relied on genomic methods, with 16S rRNA analysis proving to be a robust method for identifying and classifying the presence of a multitude of bacteria and archaea, not all of which are identifiable by more conventional methods.[12] The 16S rRNA gene, which encodes the small subunit of ribosomal RNA, is present in all DNA-based lifeforms. This gene is composed of conserved and variable regions across organisms; thus, polymerase chain reaction primers have been designed for conserved regions in order to amplify variable regions, which vary among different species.[13] Clusters of similar sequences (i.e., variable regions of amplified 16S rRNA genes that are very similar in sequence but not identical) are referred to as operational taxonomic units (OTUs) and are considered to be a particular species based on a threshold of sequence similarity to known sequences, and databases exist for this purpose.[13] Analysis of 16S rRNA has been increasingly relied on to characterize the ocular surface, particularly given its relatively low biomass.[3] We sought to (1) correlate conventional culture with microbe detection via 16S rRNA analysis, and (2) identify potential changes in the ocular microbiome induced by patients chronically taking preservative-containing eyedrops.

Methods

A cross-sectional study was performed consisting of 98 outpatients followed for management of their chronic glaucoma or for suspicion of glaucoma from the Wilmer Eye Institute's Glaucoma Center of Excellence. Participants were invited to participate during the recruitment period at routine visits and were enrolled between July 2018 and July 2019. Informed consent was obtained from each participant, and the study adhered to the tenets of the Declaration of Helsinki. Institutional review board approval was granted by Johns Hopkins.

Inclusion criteria included patients 18 years of age and older. Exclusion criteria included current ocular infection in either eye and use of any topical antibiotic eyedrop in the study eye within 6 months prior to enrollment. Each participant completed a questionnaire addressing their (1) current eyedrop regimen, (2) approximate duration of each eyedrop, and (3) use of contact lenses. Demographic information including age and sex was also collected. For each participant, the eyelid margin was swabbed using an Isohelix MS Mini DNA Swab (Cell Projects Ltd., Kent, UK) for use in 16S rRNA gene sequencing; for 49 participants, an additional sample was obtained from the eyelid margin using an ESwab (Copan Diagnostics, Murietta, CA), which was used to perform conventional bacterial culture for species identification. Two different swabs were utilized, as the Isohelix swab cannot be used to perform conventional culture. Swabs were performed by vigorously rubbing the eyelid margin of a randomly selected eye with the aforementioned swabs after instillation of one drop of 0.5% topical proparacaine for anesthesia; the Isohelix swab was performed first followed by the ESwab.

The Isohelix MS Mini DNA Swabs were sent for 16S rRNA gene deep sequencing at SeqMatic (Fremont, CA). DNA was extracted using ZymoBIOMICS DNA Miniprep Kits (Zymo Research, Irvine, CA). Sequencing libraries were generated by targeting the V4 region of the 16S rRNA gene according to the Earth Microbiome Project (EMP) protocol (http://www.earthmicrobiome.org/protocols-and-standards/16s/), which utilizes 35 polymerase chain reaction cycles. Amplicon sequencing was performed on an Illumina MiSeq System platform (Illumina, San Diego, CA) using V2 chemistry and generated 2 × 151-bp sequence reads (SeqMatic). Demultiplexing of the pooled samples was completed using the MiSeq Reporter Software System and a golay error-correcting barcode associated with the primer sequence. Raw sequence files were imported into QIIME 1.9.0 for analysis.[14] Operational taxonomic unit picking and abundance table generation were performed using the GreenGenes 16s Database (13_8) with a 97% species match threshold.[15] A negative control consisting of pure water was used by SeqMatic. For calculation of the Shannon index and Bray–Curtis dissimilarity index and to analyze the comparisons between conventional culture and 16S rRNA, the 14 most prevalent operational taxonomic units identified in the 16S rRNA analysis were used for comparative analysis. The choice of using the top 14 most prevalent OTUs rather than all microbes identified is somewhat arbitrary; however, given the high rate of contamination and erroneously identified microbes present in studies using metagenomic methods, we did not want to analyze the presence of all microbes.[16,17] Analyzing the top 14 most prevalent OTUs from each participant led to 95% of participants having their 14th most common microbe, based on OTU, account for 1.5% or less of all OTUs identified from that participant.

Fisher's exact tests were used to compare proportions of positive cultures and frequencies of commonly identified genera among participants, and Mann–Whitney *U* tests were used to compare continuous variables. The Shannon index was used to quantify diversity and was calculated in the usual fashion as the negative of the sum over each species of the proportion of each species weighted by its natural log.[18] Larger Shannon indices indicate more species diversity, and, vice versa, a Shannon index of zero would indicate only one species is present. The Bray–Curtis dissimilarity index was also calculated to quantify diversity in the usual fashion with the assumption that each participant had the same number of microbes, as this was not directly quantified by the 16S rRNA analysis. The proportion of each species was considered as its proportion of operational taxonomic unit. All statistical analysis was performed in R 4.1.3 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Ninety-eight participants were recruited with an average age ± SD of 68.6 ± 14.8 years, ranging from 22 to 97 years. Forty-four participants were women, and 54 were men. Conventional culture was performed with swabs from 49 participants, and 16S rRNA analysis was performed for all 98 participants. All conventional culture results and 16S rRNA results may be viewed online: "Supplemental Data." Thirty-one participants reported taking no eyedrops, and 67 reported taking one or more eyedrops. Ninety-five patients were able to recall their regular eyedrop regimens and were considered to be taking an eyedrop if using it daily and consistently for at least 1 month. Thirty-five participants were considered to take no preservative-containing eyedrops daily (31 denied eyedrop use, one used preservative-free cyclosporine daily, two used artificial tears sparingly only as needed, and one had been on eyedrops for less than 1 month). Twenty-two patients used only one preservative-containing eyedrop daily, and 19, 17, and two participants, respectively, used two, three, or four preservative-containing eyedrops daily (Table 1). The average number of preservative-containing eyedrops used daily was 1.2 ± 1.2 across these 95 participants. Note that combination drops containing more than one active ingredient were counted as one eyedrop (e.g., dorzolamide–timolol was counted as one preservative-containing eyedrop). Nine participants reported regularly wearing contact lenses each day during waking hours in the eye from which swabs were taken; due to this small number, differences in the microbiomes among those using and not using contact lenses were not analyzed.

Correlation Between Conventional Culture Results and 16S rRNA Genome-Based Identification

Forty-nine bacterial cultures were obtained from 49 unique participants of which 41 cultures grew bacteria and eight did not. Thirty-nine of the 41 cultures returned speciated results consisting of seven unique genera, and the remaining two cultures were reported as mixed skin flora (Table 2). Coagulase-negative *Staphylococcus* was the most commonly cultured organism, present in 34 of these 39 cultures. Various *Corynebacterium* species were the next most commonly cultured and were present in 12 of the 39 cultures (*macginleyi*, 3; *bovis*, 5; *amycolatum/striatum*, 1; *mastitidis*, 1; *tuberculostearicum*, 1; *accolens*, 1). Twenty-eight participants grew only one genus of bacteria, and 11 grew two or more. Twenty-seven participants grew only one species, and 12 grew two or more.

A total of 854 unique genera and 574 unique species were identified across all 98 participants through 16S rRNA analysis. Note that the number of genera is greater than the number of species because not all identified microbes were able to be speciated. The average numbers of unique genera and unique species identified per participant were 173 ± 61 and 84 ± 29, respectively. Among the top 14 most abundant operational taxonomic units for each participant, 309 unique

Table 1. Distribution of 95 Participants' Daily Preservative-Containing Eyedrop Regimens

Number of Participants	Number of Preservative-Containing Eyedrops Used Daily	Daily Use of Preservative-Containing Eyedrops[a]
35	0	—
22	1	17 participants took a single glaucoma drop
19	2	16 participants took two glaucoma drops
17	3	14 participants took three glaucoma drops
2	4	1 participant took four glaucoma drops

[a]Remainder of preservative-containing drops taken were artificial tears and/or steroids.

Table 2. Frequency of Bacterial Species Presence Among 39 Speciated, Culture-Positive Samples

Bacterial Species	Number of Participants With Bacterial Presence	Percent of Participants With Speciated Cultures ($n = 39$)
Coagulase-negative *Staphylococcus*	34	87.2%
Staphylococcus aureus	1	2.6%
Staphylococcus lugdunensis	3	7.7%
Corynebacterium macginleyi	3	7.7%
Corynebacterium bovis	5	12.8%
Corynebacterium amycolatum/striatum	1	2.6%
Corynebacterium mastidis	1	2.6%
Corynebacterium tuberculostearicum	1	2.6%
Corynebacterium accolens	1	2.6%
Rothia amarae	2	5.1%
Dermacoccus nishinomiyaensis	1	2.6%
Enterococcus faecalis	1	2.6%
Kocuria kristinae	1	2.6%
Microbacterium lacticum	1	2.6%

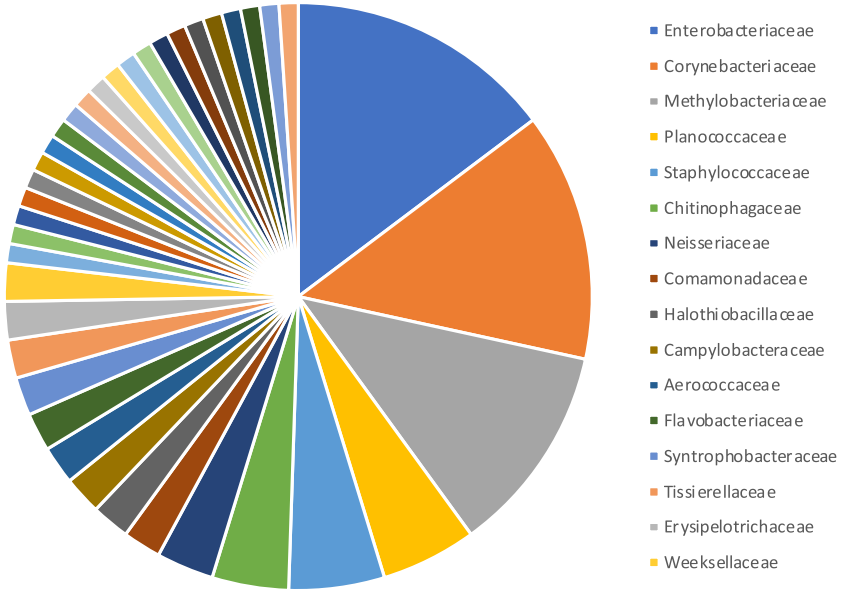

Figure 1. Single most common family across all participants.

genera and 151 unique species were identified in total across all 98 participants. The single most common family based on OTU abundance for each participant is shown in Figure 1.

To help validate microbe identification via 16S rRNA analysis, we performed intra-participant comparisons across techniques. Across all participants, 45 of the 50 (90%) non-unique genera (non-unique meaning the sum of all genera identified across all participants even if present in multiple participants) identified by conventional culture were also identified by each individual's 16S rRNA analysis within the top 14 organisms. When considering all genera identified by the 16S rRNA analysis (including organisms outside of the top 14 organisms), 48 of the 50 non-unique genera (96%) identified by conventional culture were also identified by the 16S rRNA technique. All 39 conventional culture results had one or more of their genera identified by the 16S rRNA analysis. Finally, 36 of the 39 (92%) speciated positive conventional cultures had at least one of their same genera identified within the top 14 genera detected by 16S rRNA analysis. The remaining three speciated conventional cultures had their single genus (*Staphylococcus*) identified within

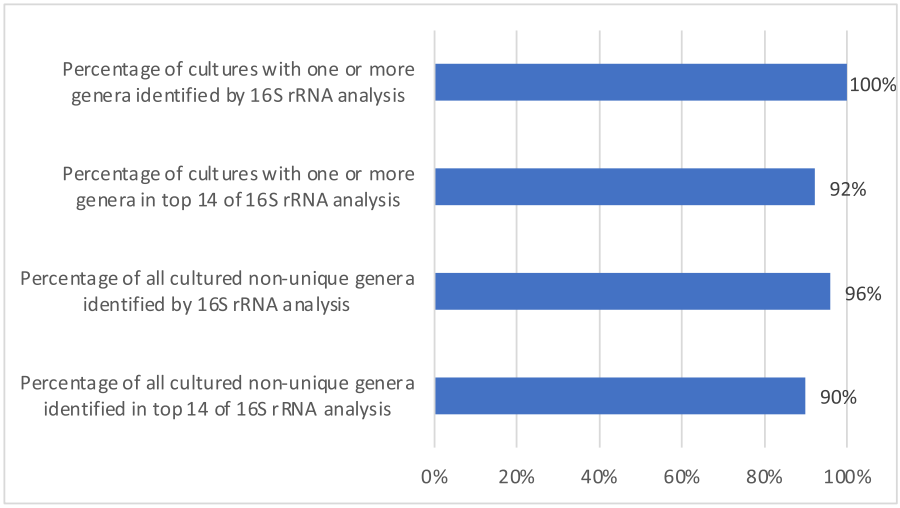

Figure 2. Cross-validation of conventional culture results compared to 16S rRNA analysis (intra-participant comparisons).

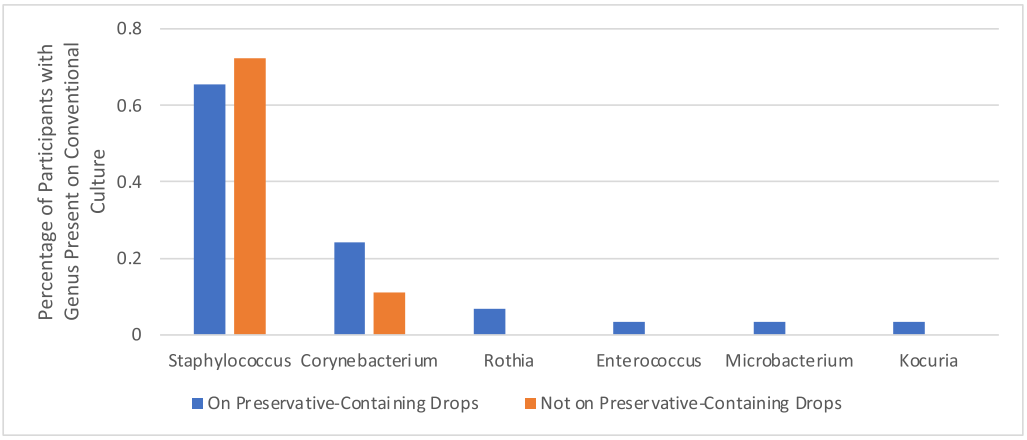

Figure 3. Percentage of participants with genera identified by conventional culture among participants using and not using preservative-containing eyedrops.

the 16S rRNA samples; however, they were not within the top 14 organisms. This may have been from slightly more skin inadvertently being sampled by the ESwab (or vice versa); moreover, it is important to remember that OTU count is not an exact marker of prevalence, and relatively more common microbes by 16S rRNA analysis are not necessarily more abundant. See Figure 2 for a graphical representation of cross-validation of conventional culture and sequencing results.

Of the 28 cultures growing only one genus, four (14%) had that genus as the most prevalent genus by relative abundance in the 16S rRNA analysis, and 15 (54%) had that genus as one of the top five most prevalent microbes identified. Of nine conventional cultures growing two genera, six (67%) and eight (89%) participants, respectively, had those same genera identified by the top 14 and by the entirety of the 16S rRNA analysis. Two participants had three genera identified by conventional culture; two of three genera were identified in both participants in the top 14, and neither participant had all three genera identified within the top 14. Of these two participants, one participant had none of their third genera (*Kocuria*) identified by 16S rRNA, and the other had all three identified by 16S rRNA analysis. We did not compare the amount of growth from conventional cultures (i.e., light vs. heavy growth) versus the relative abundance because the vast majority of species identified were classified as having very light growth (56 of 70 non-unique species among all cultures).

Correlation Between Conventional Culture Results and Preservative-Containing Eyedrop Use

Of the 49 participants who had cultures performed, 47 were able to report their eyedrop use. Twenty-nine

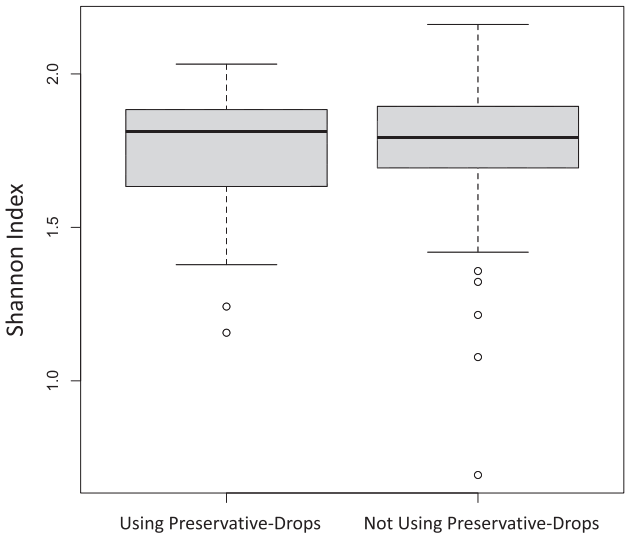

Figure 4. Shannon index for participants using and not using preservative-containing eyedrops based on 16S rRNA analysis; no statistically significant difference was observed.

participants used at least one preservative-containing eyedrop for a duration of at least 1 month. There was no difference in the bacterial culture-positive rate based on whether eyedrops were or were not being used (24/29 = 83% vs. 15/18 = 83% respectively; $P = 1$, Fisher's exact test). There was no difference in the culture positivity rate between participants who had taken at least one preservative-containing eyedrop for more than a year versus those who had taken at least one for less than a year but more than 1 month ($P = 0.70$, Fisher's exact test). Of the six genera identified by conventional culture among those 47 participants, there was no difference in the proportion of participants with a particular genus present based on whether or not preservative-containing eyedrops were used ($P > 0.05$ for all comparisons for all six genera, Fisher's exact test) (Fig. 3). Note only six genera are shown in Figure 3, as a participant with a unique genera (*Dermacoccus*) was unsure what drops they were taking and was therefore excluded from this part of the analysis.

Correlation Between 16S rRNA Analysis and Preservative-Containing Eyedrop Use

The average Shannon index was not statistically different for participants using topical preservative-containing eyedrops (1.75 ± 0.24; $n = 60$) compared to participants not using any eyedrops (1.74 ± 0.20; $n = 35$; $P = 0.82$, Mann–Whitney U test) (Fig. 4). There was also no difference in the Shannon index based on whether participants had taken a preservative-containing eyedrop for more than a year versus less than a year but still at least for 1 month ($P = 0.51$, Mann–Whitney U test). The Bray–Curtis

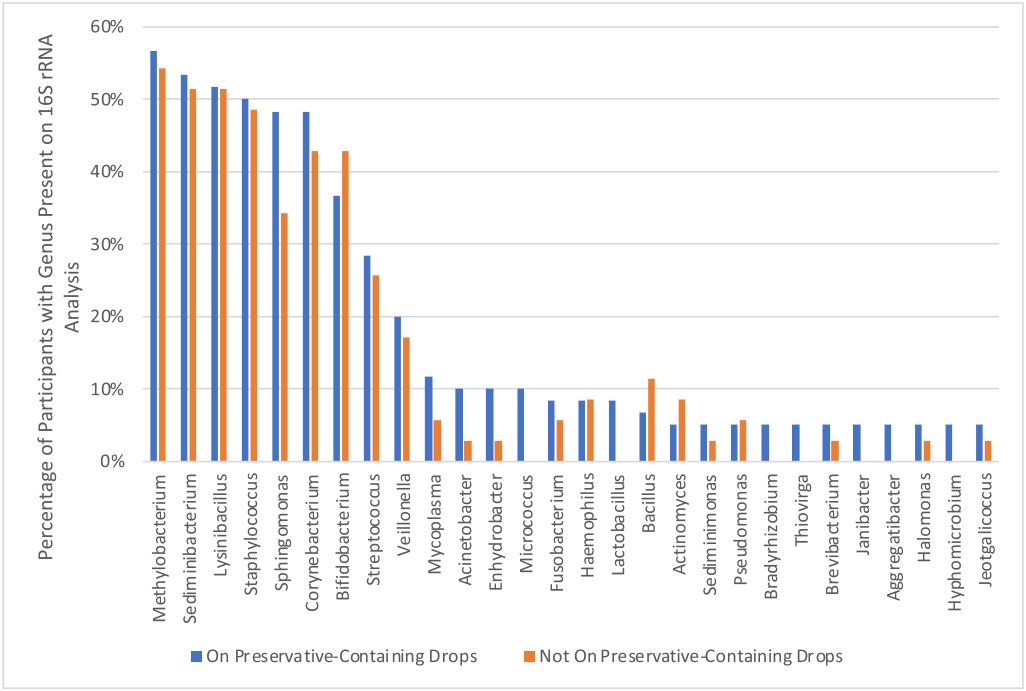

Figure 5. Percentages of participants with genera identified by 16S rRNA gene sequencing among participants using and not using preservative-containing eyedrops; no statistically significant difference in genus prevalence was found between groups.

dissimilarity index for the top 14 OTUs between the average composition of participants using versus not using preservative-containing eyedrops was 0.51, indicating that the use of eyedrops made the microbial compositions based on 16S rRNA analysis neither particularly similar nor dissimilar to one another (at the species level). There was no difference in the proportion of participants with a particular genus present based on whether or not preservative-containing eyedrops were used based on the 16S rRNA analysis ($P > 0.05$ for all comparisons, Fisher's exact test). We only analyzed genera found in at least 5% of the participants in the larger group (i.e., those using eyedrops, $n = 60$) (Fig. 5).

Discussion

Overall, the vast majority of genera detected by conventional culture of the eyelid margin of our participants were also detected by 16S rRNA gene sequencing; 90% of all non-unique cultured genera were identified within the top 14 most abundant bacteria based on 16S rRNA analysis, and 96% of them were identified by 16S rRNA analysis overall. Unsurprisingly, the number of genera identified by 16S rRNA analysis far outnumbered the number detected by conventional culture, in line with prior studies.[19] The use of preservative-containing eyedrops had no effect on the periocular microbiome composition when analyzed by conventional culture or by 16S rRNA analysis. The agreement between conventional culture data and 16S rRNA further validated the application of this technique to the periocular microbiome; however, the significance of the much larger number of organisms identified by 16S rRNA analysis remains unclear.

Validation of 16S rRNA Analysis Compared to Conventional Culture

To our knowledge, this is the first study of the ocular microbiome to correlate microbes identified by 16S rRNA analysis to those identified by conventional culture. Doan et al.[4] previously found correlation between conventional culture and microbes identified using biome representational in silico karyotyping, which relies on DNA. We found excellent ability of 16S rRNA analysis to consistently identify the genera detected via conventional culture, with 45 and 48 of the 50 genera found by conventional culture being identified within the top 14 most abundant and within all microbes identified via 16S rRNA analysis, respectively. Yet, the most common genus (*Methylobacterium*) identified among participants by 16S rRNA analysis was notably absent from conventional cultures; although this may seem disturbing, it is likely a result of the unique culture conditions required for this fastidious organism to grow.[20] The most common genera among all participants in our study (see Fig. 5)—*Methylobacterium*, *Sediminibacterium*, and *Lysinibacillus*—were not found on the eyelid margin with an average relative abundance greater than 1% by Ozkan et al.[2] However, that study recruited only 20 patients, and sampling was performed after eyelid surgery was performed and tissue was frozen. At the same time, rRNA analysis yields a far greater number of microbes identified compared to conventional culture, and the significance of their apparent presence is unclear. The average number of unique genera per participant was 173 ± 61, similar to prior studies identifying hundreds of operational taxonomic units, although prior studies have not specifically reported the number of unique genera identified.[21,22] However, to include the hundreds of genera identified by 16S rRNA analysis regardless of the relative abundance of operational taxonomic units would necessitate analyzing all microbes identified, many of which are likely spurious. Further refinements of 16S rRNA analysis with further validation by conventional methods will help determine the relevance of the larger numbers of microbes identified by 16S rRNA analysis and the threshold for defining their presence.

Lack of Alteration of Local Microbiome

The use of preservative-containing eyedrops had no effect on the lid margin microbiome detected by conventional culture methods or by 16S rRNA analysis either in terms of microbiome diversity (as quantified by the Shannon index) or proportions of participants with relatively common bacteria present. This is in contrast to Honda et al.,[9] who found a lower culture positivity rate for patients taking glaucoma eyedrops versus not (68% vs. 40%, respectively), although they found no statistically significant difference in the culture positivity rate as a function of total number of eyedrops being taken. Notably, their cultures were taken from scraping the conjunctiva directly rather than from the lid margin, as was performed in our study. Thus, it is possible that, on average, the amount of contact between eyedrops and the eyelid margin is not frequent enough or of a long enough duration to cause changes and/or that this microbiome is more robust and resistant to environmental stressors. Lim et al.[10] analyzed the eyelid microbiome via 16S rRNA

analysis of glaucoma patients naïve to versus using prostaglandin analogs and found that the microbiome was not statistically significantly altered by drop use ($P = 0.077$), similar to our study.

In general, although tools for the characterization of the ocular and periocular microbiomes continue to improve and undergo validation, the relevance of their composition and changes to it require further investigation. For example, contact lens wearers are at increased risk for several diseases; yet, Shin et al.[21] found no difference in the diversity or number of observed species of the conjunctiva or skin below the eyelid in lens wearers versus non-lens wearers, although the composition was different. With regard to trachoma, Zhou et al.[23] found decreased microbial diversity and an increased abundance of *Corynebacterium* and *Streptococcus* in patients with trachoma, although it was unclear if this had a role in pathogenesis or was a result of primary disease. Lee et al.[22] found that patients with blepharitis had less diverse microbiomes sampled from eyelashes than did healthy controls, with an increase in the relative abundance of certain bacterial species, but only 11 patients participated, and again such findings may be a result of disease rather than causative. In our own study, the clinical significance of identification of a multitude of organisms particularly with 16S rRNA analysis is unclear. For example, *Microbacterium* and *Kocuria* were identified in some participants by both techniques, and although these organisms are generally thought to be benign, they can cause serious ocular disease, including endophthalmitis.[24,25] Moreover, there was no difference in the relative proportions on average of any genus that was present in at least 5% of participants taking eyedrops by 16S rRNA analysis (Fig. 5). Thus, even for clearly pathogenic bacteria such as *Streptococcus*, the routine use of preservative-containing eyedrops did not alter their presence, although abundance may have been affected. The relevance of identifying organisms by any technique to clinical practice should be addressed by a longitudinal study examining the microbiome and correlating the incidence of ocular disease over time.

Our study has several limitations. First, drop use, including adherence and duration of use, was self-reported by participants and assumed to be accurate, yet poor adherence is well established.[26] Second, topical anesthetic was used prior to sampling to ensure patient comfort, although it has not been found to alter results of 16S rRNA analysis.[27] Finally, the swab used for 16S rRNA analysis was performed prior to swabs for conventional culture in all participants, and the impact (or lack thereof) of this approach is unknown.

Conclusions

The characterization of the ocular microbiome is augmented by 16S rRNA sequencing, but further studies are necessary to understand the significance of the large numbers of microbes identified by 16S rRNA analysis. Both techniques found no significant alteration in the periocular microbiome secondary to preservative-containing eyedrop use. Longitudinal studies relating microbiome composition to the incidence of disease development will be useful in elucidating the role of the ocular microbiome and in what ways therapeutic intervention might be indicated.

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