Paucibacterial Microbiome and Resident DNA Virome of the Healthy Conjunctiva

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PURPOSE. To characterize the ocular surface microbiome of healthy volunteers using a combination of microbial culture and high-throughput DNA sequencing techniques.

METHODS. Conjunctival swab samples from 107 healthy volunteers were analyzed by bacterial culture, 16S rDNA gene deep sequencing ($n = 89$), and biome representational in silico karyotyping (BRiSK; $n = 80$). Swab samples of the facial skin ($n = 42$), buccal mucosa ($n = 50$), and environmental controls ($n = 27$) were processed in parallel. 16S rDNA gene quantitative PCR was used to calculate the bacterial load in each site. Bacteria were characterized by site using principal coordinate analysis of metagenomics data. BRiSK data were analyzed for presence of fungi and viruses.

RESULTS. *Corynebacteria, Propionibacteria*, and coagulase-negative *Staphylococci* were the predominant organisms identified by all three techniques. Quantitative 16S PCR demonstrated approximately 0.1 bacterial 16S rDNA/human actin copy on the ocular surface compared with greater than 10 16S rDNA/human actin copy for facial skin or the buccal mucosa. The conjunctival bacterial community structure is distinct compared with the facial skin ($R = 0.474$, analysis of similarities $P = 0.0001$), the buccal mucosa ($R = 0.893$, $P = 0.0001$), and environmental control samples ($R = 0.536$, $P = 0.0001$). 16S metagenomics revealed substantially more bacterial diversity on the ocular surface than other techniques, which appears to be artifactual. BRiSK revealed presence of torque teno virus (TTV) on the healthy ocular surface, which was confirmed by direct PCR to be present in 65% of all conjunctiva samples tested.

CONCLUSIONS. Relative to adjacent skin or other mucosa, healthy ocular surface microbiome is paucibacterial. Its flora are distinct from adjacent skin. Torque teno virus is a frequent constituent of the ocular surface microbiome. (ClinicalTrials.gov number, NCT02298881.)

Keywords: microbiome, virome, conjunctiva

Human mucosal surfaces including the gastrointestinal tract, oral mucosa, and respiratory tract are normally colonized by a diverse community of microorganisms. Alterations of the native microbiome may lead to pathogenic infections or inflammation in the host.[1-4] As the ocular surface includes the mucosal tissues of the bulbar conjunctiva, conjunctival fornix, and palpebral conjunctiva, an important question in ophthalmology has become whether the ocular surface hosts a resident microbiome that is similarly as diverse as other mucosal surfaces.[5]

A complete cataloging of the ocular surface microbiome has significant implication in clinical ophthalmology. It is known that many ocular infections such as postoperative endophthalmitis, blebitis, and infectious keratitis originate from the ocular surface. However, a definitive diagnosis of the causative pathogen can be challenging. Culture yields for infectious-appearing corneal ulcers are typically less than 60%.[6,7] Similarly, in postoperative endophthalmitis (where there is little doubt of an infectious etiology), the success rate of pathogen identification by conventional bacterial culture is

only approximately 50 to 70%.[8,9] In a recent study, cultures failed to detect a causative agent in approximately 33% of the postoperative endophthalmitis cases, while biome representational in silico karyotyping (BRiSK; a metagenomic detection method for DNA-based life-forms) was able to identify a small DNA virus, torque teno virus (TTV), in 100% of culture-negative endophthalmitis samples.[10] These results suggest that TTV may be an overlooked or unknown resident virus of the ocular surface and further support the importance of understanding both the bacterial and viral constituents of the normal ocular surface.

While numerous studies conducted over the past 50 years have examined the flora of the ocular surface using traditional culture techniques, it is clear that such techniques have detected only a subset of species present on the ocular surface.[5,11,12] Recently, several investigators have used molecular metagenomics techniques to characterize the organisms on the ocular surface, finding that the ocular surface bacterial flora appears more diverse than previously characterized using culture techniques.[11,13-15] In one study where the

researchers compared organisms identified with conventional cultures to broad range 16S rDNA gene PCR, cultures only identified *Staphylococcus* species and *Bacillus*, while molecular techniques identified five more genera including several uncultured bacteria.[14] Similarly, Dong et al.[13] used 16S rDNA gene deep sequencing to study the ocular surface of four healthy individuals, and were able to detect DNA corresponding to 59 distinct bacterial genera. Although these results demonstrate potential diversity of the ocular surface microbiome, these studies examined a relatively small number of individuals, and did not assay for nonbacterial organisms or viruses, as 16S rDNA gene sequencing cannot detect other classes of microorganisms such as viruses or fungi. Previous work using viral-directed PCRs have shown that herpes simplex type 1, hepatitis B virus, and even hepatitis C virus can be detected in the tears of asymptomatic volunteers,[16] suggesting a potential niche for viruses in the ocular surface microbiome. Therefore, simply focusing solely on the bacterial constituents of the ocular surface may result in an incomplete understanding of the ocular surface microbiome.

In the current study, we carried out a comprehensive analysis of the ocular surface microbiome in over 100 healthy volunteers using traditional culture, 16S rDNA gene qPCR and deep sequencing, and BRiSK. This is among the largest studies to date using high-throughput DNA sequencing technologies to characterize of ocular surface microbiome. We find that that the ocular surface supports a relatively consistent, paucibacterial microbiome. We also find that a majority of healthy individuals have a resident ocular surface virome with TTV being the predominant virus.

METHODS

Conjunctival Swab Collection

This prospective study was approved by the University of Washington Human Studies Division institutional review board (Seattle, WA, USA). Research adhered to the tenets of the Declaration of Helsinki and was conducted in accordance with Health Insurance Portability and Accountability Act regulations. The study was registered with clinicaltrials.gov (NCT02298881). Written informed consent was obtained from all subjects. Participants were enrolled from a combination of clinical ophthalmology offices, medical school students, and seniors living in retirement communities. Subjects were given a questionnaire assessing their systemic health including questions regarding immunocompromised states such as diabetes and HIV status. Ocular and systemic medication use over the past year were queried, including use of corticosteroids and immunomodulatory drugs. The ocular surface disease index (OSDI) questionnaire was used to identify subjects with healthy ocular surface, using score of 6 or under as the cutoff point. Enrolled subjects were ages 18 to 30 or 60 and older (in order to use these same groups in subsequent studies of the effect of contact lens wear and cataract surgery, respectively, on the surface microbiome). Exclusion criteria were obvious ocular surface disease/irritation, facial skin disease, history of recent contact lens wear, use of oral or topical antibiotics or prescription eye medication in the past 3 months, ocular surgery in the last 12 months, active ocular infection, dry eye condition, diabetes, or immune-compromised state. After instillation of sterile, topical proparacaine, DNA swabs (SK-2S; Isohelix, Boca Raton, FL, USA) were used to obtain samples from the superior and inferior fornices of both eyes for molecular analysis. In a subset of subjects, swabs were taken from buccal mucosa and/or cheek skin. All swabs were immediately placed on ice and transferred to lab within 6 hours to be stored at −80°C. Environmental 'air swabs' consisting of exposing freshly-opened swabs to air in proximity of the subject were collected at same time as subject collections for each fourth subject (27 total), and treated identically to subject swabs.

Cultures

Calcium alginate swabs (25-800-A-50; Puritan, Guilford, ME, USA) were used to prepare culture samples. Each swab was directly plated onto chocolate, blood, and Sabaraud's agar plates, and then placed into thioglycolate broth. Plates were incubated at 35°C aerobically, anaerobically, or under elevated CO_2 per standard microbacterial laboratory protocol. Colonies were enumerated and microbes were identified using standard biochemical methods. All plates and broth were incubated for 30 days to isolate slow growing species.

DNA Purification

Genomic DNA was isolated from the conjunctiva swabs using the DNeasy Blood & Tissue Kit (Qiagen, Inc., Venlo, Netherlands) as per protocol. The DNA was eluted in 30 uL of the kit elution buffer and stored at −20°C. DNA was quantified using the Qubit dsDNA HS Assay Kit (Thermo-Fisher Scientific, Waltham, MA, USA). In cases with limited DNA recovery or quality, priority was given to 16S metagenomics sequencing first (89 subjects) and BRiSK second (80 subjects).

Broad Range 16S rDNA Gene, β-Human Actin, and TTV Quantitative Polymerase Chain Reactions

These methods were performed as described previously.[10] Pan bacterial PCR was performed using 16S ribosomal DNA universal primers (Integrated DNA Technologies, San Diego, CA, USA). The sequences of the primers were 5′-GAG GAAGGTGGGGATGACGT-3′ and 5′-AGGCCCGGGAACGTATT CAC-3′. Quantitative PCR (qPCR) assay was performed on the Applied Biosystems (Foster City, CA, USA) 7500 Fast Real-Time PCR system. The final PCR mix contained 0.8 μL each of forward and reverse primers (final concentration of each, 0.4 mM), 10 μL of the FastStart SYBR Green Master Mix without ROX (Roche, Basel, Switzerland), and 2 μL of unamplified genomic DNA. Master mix was prepared under a laminar hood to minimize contaminations. The final reaction volume was 20 μL. The primer pairs for TTV PCR were 5′-AGGTGAGTTTACACACCGCAGTCA-3′ and 5′-AATGAAGACCC TAAGAGCCTTGCC-3′. The primers for β-actin were 5′-TGCTCCTCCTGAGCGCAAG-3′ and 5′-GCCGGACTCGTCA TACTCC-3′. The final PCR mix was performed in a similar fashion as the broad range 16S rDNA gene PCR, but with Absolute Blue qPCR SYBR low ROX Mix (Thermo Fisher Scientific) instead of FastStart SYBR Green Master Mix. For standard curve, a plasmid complementary DNA of the cloned gene of interest (e.g., the target sequence for 16S, TTV, or actin) was serially diluted 10-fold to obtain copy numbers ranging from 1×10^1 to 10^8 copy/mL. Quantitative PCR routinely was able to detect 10 copies/mL of each control complementary DNA. All samples were performed in duplicate. The run consisted of initial holding stages at 50°C for 2 minutes and at 95°C for 10 minutes, followed by a cycling stage (25–28 cycles) at 95°C for 15 seconds and 60°C for 1 minute. Copy number of experimental samples was calculated by interpolation of delta C_T number against the standard curve derived from the cloned product.

Biome Representational In Silico Karyotyping (BRiSK) Technique

BRiSK is a representational deep sequencing technique that allows for an unbiased characterization of the DNA-based metagenomic constituents in biopsy samples.[17] BRiSK uses a Type IIB DNA restriction enzyme to create a defined representation of 27-mer DNAs in a sample. Massively parallel sequencing of this representation allows for construction of high-resolution karyotypes and identification of multiple species within a biome using BRiSK software. Briefly, genomic DNA was subjected to whole genome amplification using phi29 and digested with BsaXI as previously described. Illumina-compatible barcoded paired-end adapters, one of which is biotinylated, were ligated to digested DNA, followed by isolation of sequences with asymmetric adapters using streptavidin column and PCR-amplified using adapter sequences. The resulting product was gel-purified and subjected to high throughput sequencing on the Illumina MiSeq sequencer (Illumina, San Diego, CA, USA). Obtained sequence reads were analyzed using BRiSK software, which performs parsing and matching against an SQL database containing all tags resulting from a virtual BsaXI digest of all sequences from GeneBank divisions of primates, bacteria, invertebrates, phages, and viruses.

Illumina V3-V4 16S Library Construction and Data Processing

The ocular surface bacterial community was assessed by deep sequencing the V3-V4 hypervariable regions of the 16S rDNA gene. Library preparation was performed per Illumina16S metagenomic sequencing library preparation protocol.[18,19] The sequences of the amplicon primers were 5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCC TACGGGNGGCWGCAG-3′ and 5′ GTCTCGTGGGCTCGGA GATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′.[20] This region of the 16S rDNA gene appears optimal for interrogating bacterial communities.[21] Briefly, the V3-V4 region was amplified using a two-step PCR strategy. The first PCR was conducted using the amplicon primers (from 5′–3′) that contain the Illumina overhang adaptor and the universal V3-V4 priming sequence. Each reaction mixture contains 5 ng of extracted DNA and KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Wilmington, MA, USA) in a final volume of 25 uL. The PCR conditions involved a denaturation step at 95°C for 3 minutes followed by an initial denature step at 95°C for 30 seconds, an annealing step at 55°C for 30 seconds, and an extension step at 72°C for 30 seconds for a total of 25 cycles followed by a final elongation at 72°C for 5 minutes. The resulting 16S V3-V4 amplicons were purified using AMPure XP beads (Beckman Coulter, Brea, CA, USA). Of the purified first PCR products, 5 uL were used in a 50 uL PCR using the Nextera XT Index Kit (Illumina, San Diego, CA, USA) to attach dual indices and Illumina sequencing adaptors. The PCR conditions involved a denaturation step at 95°C for 3 minutes followed by an initial denature step at 95°C for 30 seconds, an annealing step at 55°C for 30 seconds, and an extension step at 72°C for 30 seconds for a total of eight cycles followed by a final elongation at 72°C for 5 minutes. The resulting amplicons were purified with AMPure XP beads to clean up the final library before quantification. The DNA concentration in each reaction was quantified using the Qubit dsDNA HS Assay Kit (ThermoFisher Scientific) according to instructions. Finally, equal DNA amounts of all samples were pooled to a final concentration of 4 nM and subjected to paired-end Illumina sequencing on the MiSeq using paired 300-bp reads and MiSeq v3 reagents. Addition of random PhiX phage DNA

to the solution (10%) provided calibration and cluster generation on the MiSeq's flow cell. The samples were analyzed using the BaseSpace 16S Metagenomics App, which is a pipeline that uses the full GreenGenes reference database.[22]

Statistics

All analyses were implemented in the R software environment (in the public domain, http://cran.r-project.org/), version 3.1.3. Normalized 16S values were compared using the Wilcoxon rank test (for skewed data) and Kruskal-Wallis with Dunn's test for multiple comparisons.

Bacterial community structures were visualized by using Bray-Curtis nonmetric multidimensional scaling (NMDS) using all sequences obtained from subjects in all tested groups. The analysis of similarities (ANOSIM) was used on the distance matrix to determine if any groups of samples contained significantly different bacterial communities. *P* values equal or above 0.001 were considered for significance because of adjustment for multiple comparisons. The *R* values of the statistical test ANOSIM provide an estimate of the effect size and range from 1 to −1. *R* values closer to 0 indicate that no differences exist between the groups (controls, conjunctiva, skin, buccal mucosa), whereas values closer to 1 indicate that differences between the various groups exist.

The differential expression of specific bacterial genera between groups was determined using the two-stage statistical procedure.[23] This was a statistical algorithm developed by Pookhao et al.[23] to detect differentially abundant features in large metagenomes under various conditions. In the first stage, the algorithm aims to detect informative features associated with a particular phenotype that results in the dimensional reduction of the metagenomic data. In the second stage, the algorithm aims to detect features that are statistically differentially abundant in two or more conditions by using the negative binomial model with the resulting *P* values corrected for multiple comparisons using the Benjamini and Hochberg's false discovery rate[24] at significance level of 0.05.

RESULTS

Bacterial Culture

A total of 107 subjects participated in this study. Sixty-two subjects (31 male and 31 female) were age 30 or less; of these, 47 were Caucasian, 1 was African-American, 11 were Asian, and 3 self-classified as 'other'. Of the 45 subjects in the 60 and older age group (21 male and 24 female), 38 were Caucasian, 4 were African American, and 3 were Asian. Conventional bacterial cultures were used to detect the presence of microbes in 428 conjunctiva samples from the 107 healthy adult subjects (Table 1). In total, 78.5% (*n* = 336) of all conjunctiva swab samples were positive for growth of at least one organism. Coagulase-negative *Staphylococcus* species were the most frequently isolated organisms at 45.3% followed by *Propionibacterium* at 33.9% and *Diphtheroids* at 15.4%. Other organisms identified in a small number of conjunctiva swabs were *Streptococcus viridans*, *Micrococcus*, *Bacillus*, unidentified Gram-positive rods, *Neisseria*, unidentified Gram-positive cocci, and *Hemophilus*. *Rothia mucilaginosa*, *Mycelia sterilia*, *Lactobacillus*, *Rhodotorula*, *Escherichia coli*, *Klebsiella pneumonia*, *and Moraxella* were identified once each. In addition to bacterial species, *Aspergillus* and other molds were also infrequently detected.

TABLE 1. Microbial Flora Isolated From 428 Healthy Conjunctiva Samples ($n = 107$) With Conventional Cultures

Organisms	N (%)
No growth	92 (21.5)
Gram-positive bacteria	
Coagulase-negative staphyloccocus	194 (45.3)
Propionibacterium	145 (33.9)
Diphtheroids	66 (15.4)
Streptococcus	15 (3.5)
Coagulase-positive staphylococcus	9 (2.1)
Micrococcus	9 (2.1)
Bacillus	9 (2.1)
Lactobacillus	1 (0.2)
Rothia	1 (0.2)
Unidentified	11 (2.5)
Gram-negative bacteria	
Neisseria	4 (0.9)
Hemophilus	2 (0.5)
Escherichia	1 (0.2)
Enterobacter	1 (0.2)
Moraxella	1 (0.2)
Fungi	
Mycelia	1 (0.2)
Rhodotorula	1 (0.2)
Aspergillus	1 (0.2)
Unidentified	2 (0.5)

Some samples yielded more than one organisms. All cultures were incubated for 30 days.

Healthy Human Conjunctiva is Paucibacterial Relative to Buccal Mucosa or Skin

The presence of bacterial DNA in conjunctiva samples was quantified using conventional broad-range 16S rDNA gene qPCR using a calibrated control template for quantification (Fig. 1). Human β-actin DNA was also quantified by qPCR to control for sample loading. In addition to collecting samples from the conjunctiva of healthy adults, samples from the facial skin and the buccal mucosa from a subset of volunteers were also obtained (Fig. 1A). The normalized bacterial load for all conjunctival samples was 0.14 ± 0.03 16S rDNA copies/human actin copy (mean $\pm$ SEM, $n = 82$), which was approximately 150-fold less than the normalized bacterial load of the buccal mucosa (24.29 ± 8.08 16S/human actin copy, $n = 28$) or the facial skin (33.51 ± 6.96 16S/human copy, $n = 25$). These results translated to approximately 0.06 bacterium per human cell in the conjunctiva compared with approximately 12 bacteria per human cell in the buccal mucosa and approximately 16 bacteria per human cell in the skin, assuming an average of 4.2 16S rDNA gene copies per bacterial genome.[25]

The lower conjunctiva contained more bacterial DNA than the upper conjunctiva (0.20 ± 0.05 16S/human actin copy, mean $\pm$ SEM, $n = 82$ vs. 0.11 ± 0.03, $n = 79$, respectively; $P = 0.02$, Wilcoxon rank sum test; Fig. 1B). While there was no statistically significant difference in the bacterial load between women versus men (0.18 ± 0.06, $n = 37$ vs. 0.08 ± 0.02, $n = 45$, $P = 0.86$; Fig. 1C), there was a statistical significant difference in the bacterial load between subjects older than 60-years old and subjects younger than 30-years old (0.16 ± 0.04, $n = 39$ vs. 0.12 ± 0.05, $n = 149$, $P = 0.005$; Fig. 1D). Figure 2 showed that the bacterial load was not dependent on laterality, between the right and left eyes within subjects. There was no statistical difference in bacterial DNA between the upper eyelids of the right and left eyes (0.11 ± 0.03 vs. 0.12 ± 0.03, $n = 77$, $P = 0.32$; Fig. 2A) or between the lower eyelids of the

right and left eyes (0.18 ± 0.05 vs. 0.56 ± 0.03, $n = 73$, $P = 0.79$; Fig. 2B).

16S rDNA Gene Deep Sequencing Reveals Diversity in the Ocular Surface Microbiome

Upper and lower conjunctiva of both eyes ($n = 356$), facial skin ($n = 42$), and buccal mucosa ($n = 50$) swab samples were collected from 89 healthy subjects along with environmental samples ($n = 27$) for 16S rDNA gene deep sequencing. Of the subjects, 49 were younger than 30-year old and 40 subjects were older than 60-years old. Of these subjects, 46 were women and 43 were men. 16S rDNA gene deep sequencing of these samples yielded a total of 133,272,558 sequence reads with 75.6% total reads remaining following post sequencing quality measures, resulting in an average read depth per sample of $201,410 \pm 267,540$. Among these sequences, 71% of the total number of classified reads across samples were represented by relatively few genera. *Pseudomonas, Elizabethkingia, Corynebacterium, Staphylococcus, Delftia, Propionibacterium*, and *Streptococcus* predominated in conjunctival samples, comprising nearly 63.6% of the total number of classified reads across the conjunctiva samples. Of the reads, 28.4% passing quality control were unclassified.

Although this relatively small number of genera predominated, sequences from over 800 genera were identified. For conjunctiva, on average, 245 different genera were identified per sample using 16S rDNA gene deep sequencing (range, 17–579). This finding raises the strong possibility that some of these are artefactual. Production of DNA-free reagents is challenging, and false-positive amplifications are common.[26,27] Quantitative PCR of the bacterial load in the conjunctiva showed that there is approximately 1 bacterium for every 20 human conjunctival epithelial cells collected on conjunctival swab. Based on this, we estimate there should be approximately 38 bacteria detected per conjunctival sample given that one diploid human genome weighs approximately 6.6×10^{-12} g and 5×10^{-9} g of DNA was processed for each 16S PCR sample. A reasonable assumption is that the majority of the genera identified are contaminants during sample processing. To assign a confidence measure to determined differences, we compared the bacteria recovered from conjunctival swabs with those of the environmental control. To determine those bacteria likely to show meaningful enrichment in conjunctiva, we first limited our analysis to those bacteria whose total 16S counts constituted 0.1% or greater of the conjunctival microbiome. This analysis reduced the number of genera from 887 to 42. We next employed a novel heuristic to rank these bacteria for 'confidence'. We reasoned that confidence in a particular result would be a function of the magnitude of fold difference in the observed bacteria between conjunctiva and environmental samples, and the P value for the difference between these sites (adjusted for multiple comparisons, see Methods for details of calculation of P values). Because of the large number of samples analyzed, P values for significant differences included 17 that were smaller than 1×10^{-10}. The confidence heuristic was calculated as $\log_{10}$(fold difference/adjusted P value), where the fold difference was mean conjunctival count/mean environmental count (Table 2). Four of six highest confidence genera were *Corynebacteria, Propionibacteria, Staphylococcus,* and *Streptococcus*, which were all detected frequently in cultures and had confidence heuristics of greater than 10. However, *Pseudomonas* and *Elizabethkingia*, which conjointly accounted for 29% of all reads in conjunctiva, had confidence levels of 2 or less, a difference of over 10 orders of magnitude from these other bacterial genera. Examination of
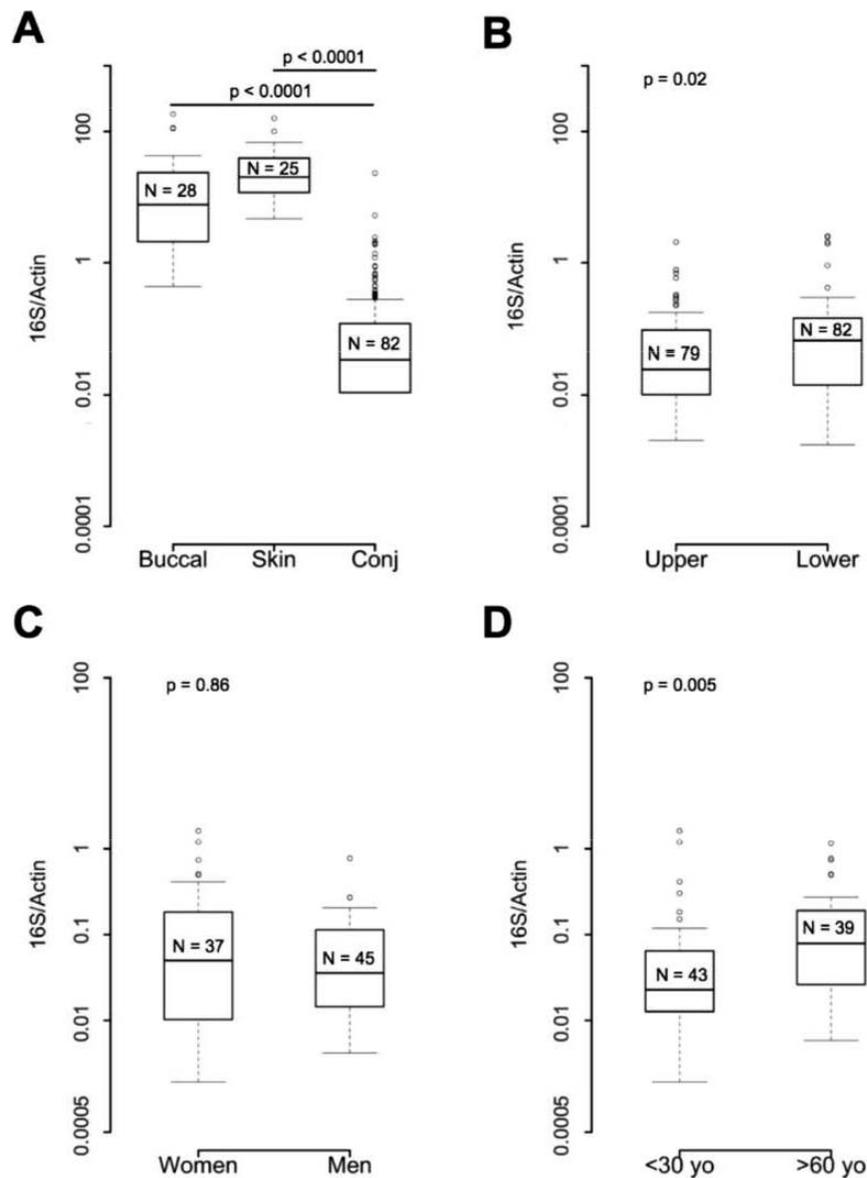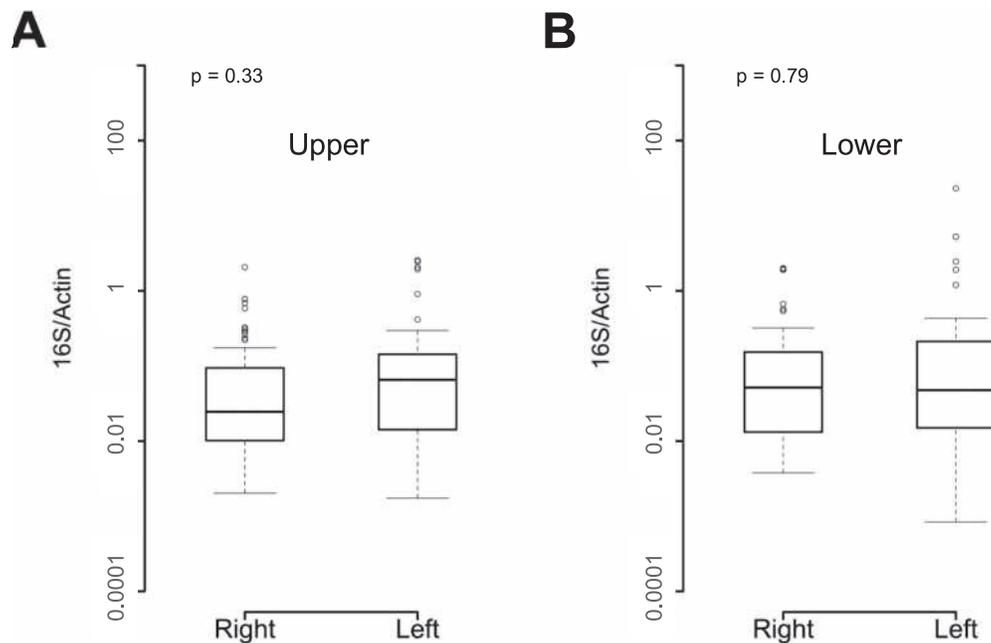
Figure 1. Quantification of 16S rRNA gene in healthy subjects. Detection of 16S rRNA gene normalized to human β-actin in (**A**) the conjunctiva comparing with the buccal mucosa and the facial skin, (**B**) the fornix of the upper eyelid compared with the lower eyelid, (**C**) the conjunctiva between men and women, and (**D**) the conjunctiva in subjects younger 30 years and older than 60 years. *P* values were calculated using Wilcoxon rank test and Kruskal-Wallis with Dunn's test for multiple comparisons.

the distribution of confidence measures across the 42 genera (Fig. 3) reveals a remarkably broad distribution, with 22 genera having confidence heuristics less than 5, while 17 genera have confidence heuristics at least 5 logs higher (i.e., ≥ 10). This latter set includes a number of genera detected by culture in some samples (e.g., *Lactobacillus* and *Haemophilus*), as well as bacteria not detected by culture including *Anaerococcus, Parascardovia, Peptoniphilus, Actinomyces, Prevotella, Dietzia, Finegoldia, Dermacoccus, Fusobacterium, Porphyromonas,* and *Mycobacterium*. However, none of these species accounted for more than 0.8% of total conjunctival reads. Thus, we find the core cluster microbiome identified by deep DNA sequencing consists of the same four predominant species as detected by culture: *Corynebacteria, Propionibacteria, Staphylococcus,* and *Streptococcus,* likely with sporadic or very low amounts of several other species.

Community Structure Comparison by Body Sites

We compared NMDS plots (a method of principal coordinate analysis) using all V3-V4 sequences from samples collected from the conjunctiva, buccal mucosa, and facial skin of healthy subjects (Fig. 4). Control samples were considered as the environmental swabs. Nonmetric multidimensional scaling plot provides a two-dimensional visualization of differences between members of a population, with similar members grouping together. This analysis allows measurement of overall differences in microbial populations among the various sample groups. The bacterial communities of the healthy conjunctiva clustered differently than the skin ($R = 0.474$, ANOSIM $P = 0.0001$) and also differently than the buccal mucosa ($R = 0.893$, $P = 0.0001$). Importantly, there existed a statistically significant difference in the communities between the healthy conjunctiva and the control environmental samples ($R = 0.536$, $P = 0.0001$). Together, these results indicate that while the facial

Figure 2. Quantification of 16S rRNA gene between the left and right eyes in healthy subjects. No statistical significant difference existed in the normalized 16S rRNA gene to human β-actin in (**A**) the upper eyelids ($N = 77$), and (**B**) the lower eyelids ($N = 73$) of the right and left eyes. *P* values were calculated using Wilcoxon rank test and Kruskal-Wallis with Dunn's test for multiple comparisons.

skin, the oral mucosa, and the ocular surface are adjacent sites, the microbiome of each site is unique.

BRiSK Analysis Reveals Viruses on the Ocular Surface of Most Healthy Subjects

BRiSK is a technique for characterizing all DNA present in a sample using a Type IIB restriction enzyme (BsaXI) that yields a 27 bp double-stranded restriction product. These small DNA fragments are then sequenced up to 10-fold coverage. The advantage of this technique is that it allows for the identification of all abundant organisms present in a complex clinical sample, including viruses, fungi, and parasites. Eighty samples of the left lower eyelid from 80 healthy subjects were subjected to BRiSK sequencing and analysis. The results are shown in Figure 5. Of the 78 organisms identified, 10 were detected in 86.3% of all subjects. These organisms included *Corynebacterium, Propionibacterium, Staphylococcus, Streptococcus,* Multiple Sclerosis–associated retrovirus (MSRV), Human Endogenous Retrovirus K (HERV-K), *Paenibacillus*, *Clostridium*, TTV, and *Bacillus* (Fig. 5A). Consistent with culture and 16S rDNA gene deep sequencing, *Propionibacterium*, *Corynebacterium*, *Streptococcus*, *and Staphylococcus* predominated. Notably, BRiSK also revealed the presence of multiple viruses in the conjunctiva. Multiple Sclerosis–associated retrovirus, HERV-K, and TTV were the top three viruses identified. As both MSRV and HEV-K are human endogenous retroviruses (HERV), the DNA sequences detected here likely originated from the host DNA, and hence their clinical significance is unclear.[28] The presence of TTV on the ocular surface is particularly noteworthy. Torque teno virus is a 3.8 kb, single-strand circular DNA anellovirus that has been associated with seasonal hyperacute panuveitis (SHAPU) and culture-negative endophthalmitis.[10,29] To confirm the presence of TTV on the healthy ocular surface, directed quantitative PCR in healthy conjunctiva samples was performed. Of 80 tested samples, 52 harbored at least 1 TTV copy per 100 epithelial cells (Fig. 5B). Subgroup analyses revealed no significant

differences between men and women (0.079 ± 0.068 and 0.014 ± 0.005 TTV/human actin copy, mean ± SEM; *P* = 0.547) or between subjects younger than 30 years and older than 60 years (0.075 ± 0.059 and 0.008 ± 0.002, *P* = 0.089). Additional confirmation of the presence of TTV was performed by visualizing the amplified TTV products on agarose gels. Figure 5C demonstrates the presence and absence of TTV in a subset of healthy conjunctiva samples. In addition to TTV, sequences matching Merkel Cell Polyomavirus (MCV), human papillomavirus (HPV), and Abelson murine leukemia virus were detected in two samples each by BRiSK. Given that these viruses were detected in only a few healthy subjects, their significance is unclear although MCV and HPV are known to be present in healthy epithelial cells.[30,31] Together, the BRiSK analysis of the healthy conjunctiva suggests that there exists a resident DNA viral community on the ocular surface of most subjects with TTV predominant.

DISCUSSION

Unlike the gastrointestinal tract, oral mucosa, vaginal mucosa, respiratory tract, and skin, where next-generation sequencing techniques have been extensively applied to understand native microbial communities, the ocular surface microbiome has been infrequently studied using these techniques.[1,32–34] To establish a more definitive characterization of the ocular surface microbiome, we applied three complementary techniques (traditional culture, 16S rDNA gene deep sequencing, and BRiSK) to detect the microbial and other DNA-based communities in the conjunctival swab samples of 107 healthy volunteers. To our knowledge, this study is among the largest studies to date using cultivation-independent techniques to survey the constituents of the ocular surface microbiome. Consistent with previous studies,[11,12,14] few organisms were identified using conventional cultures. Quantification of bacteria load from various body sites using 16S rDNA qPCR revealed that the conjunctiva is truly paucibacterial, harboring approximately 150- to 200-fold fewer bacteria per human cell

TABLE 2. Descriptive Statistics for the 42 Genera Whose 16S Read Counts Were Greater Than or Equal to 0.1% of the Conjunctival Microbiome

Annotation	Environment Mean	Conjunctiva Mean	Fraction of Total Conjunctiva, %	Adjusted *P* Value	Fold Difference, Conj/ENV	log Confidence
Corynebacterium	104.48	15435.04	14.20	1.33E−48	147.73	50.05
Anaerococcus	1.33	882.73	0.81	5.92E−32	665.96	34.05
Propionibacterium	335.53	8960.10	8.24	8.15E−33	26.70	33.52
Parascardovia	1.05	247.37	0.23	1.28E−25	236.50	27.27
Streptococcus	323.78	4770.05	4.39	1.28E−24	14.73	25.06
Staphylococcus	409.23	14352.08	13.20	3.95E−21	35.07	21.95
Peptoniphilus	11.25	708.21	0.65	1.32E−20	62.96	21.68
Lactobacillus	8.90	256.69	0.24	2.03E−20	28.84	21.15
Actinomyces	8.01	208.27	0.19	7.21E−20	25.99	20.56
Prevotella	10.24	487.10	0.45	1.05E−18	47.56	19.66
Dietzia	8.18	291.44	0.27	3.50E−17	35.62	18.01
Finegoldia	1.50	432.88	0.40	6.87E−16	288.36	17.62
Haemophilus	23.72	416.19	0.38	1.07E−16	17.55	17.21
Dermacoccus	0.53	126.19	0.12	7.53E−15	239.64	16.50
Fusobacterium	4.73	126.97	0.12	2.00E−12	26.82	13.13
Porphyromonas	10.84	267.73	0.25	2.08E−12	24.69	13.08
Mycobacterium	12.03	130.32	0.12	5.87E−12	10.83	12.27
Granulicatella	10.88	223.74	0.21	9.07E−09	20.56	9.36
Gemella	43.85	297.05	0.27	1.60E−07	6.77	7.63
Microbacterium	73.95	285.82	0.26	2.42E−07	3.86	7.20
Mycoplasma	61.74	245.88	0.23	2.09E−05	3.98	5.28
Acinetobacter	127.73	603.43	0.55	2.98E−05	4.72	5.20
Burkholderia	36.88	128.50	0.12	7.31E−05	3.48	4.68
Candidatus Phytoplasma	114.37	417.61	0.38	1.55E−04	3.65	4.37
Devosia	98.59	321.83	0.30	4.91E−04	3.26	3.82
Brevundimonas	389.54	732.80	0.67	4.07E−04	1.88	3.67
Pedobacter	704.03	1948.33	1.79	8.93E−04	2.77	3.49
Sphingomonas	1465.29	541.74	0.50	1.35E−04	0.37	3.44
Flavobacterium	513.87	1092.55	1.00	1.36E−03	2.13	3.19
Myroides	294.28	603.68	0.56	3.39E−03	2.05	2.78
Treponema	66.59	193.83	0.18	6.44E−03	2.91	2.66
Micrococcus	29.89	119.48	0.11	9.22E−03	4.00	2.64
Bifidobacterium	76.91	173.23	0.16	1.05E−02	2.25	2.33
Agrobacterium	3977.58	2344.09	2.16	4.53E−03	0.59	2.11
Lysinibacillus	1999.40	304.70	0.28	1.30E−03	0.15	2.07
Pseudomonas	19304.40	30263.02	27.83	1.43E−02	1.57	2.04
Ralstonia	502.46	1047.92	0.96	2.10E−02	2.09	2.00
Mesorhizobium	557.18	205.60	0.19	4.32E−03	0.37	1.93
Elizabethkingia	7844.87	12324.85	11.34	3.34E−02	1.57	1.67
Saccharopolyspora	56.79	120.43	0.11	4.76E−02	2.12	1.65
Chryseobacterium	2111.52	3122.79	2.87	3.86E−02	1.48	1.58

than the facial skin or the buccal mucosa. The community of the conjunctiva appears to be uniquely constituted compared with either skin or buccal mucosa, and contains both resident bacterial and often resident viral communities. The core constituents of the conjunctival microbiome appear relatively consistent between individuals, and are dominated by the four genera of coagulase-negative *Staphylococci*, *Diphtheroids*, and *Propionibacteria*, and *Streptococci*. Analysis using BRiSK demonstrated that a majority of healthy subjects have detectable TTV on the ocular surface, which was confirmed by direct PCR.

It has long been apparent that cultures of the ocular surface typically yield relatively few bacteria.[11,14,35,36] These organisms are predominantly coagulase-negative *Staphylococcus*, diphtheroids, and *Propionibacterium acnes*.[11,14,35,36] However, recent analyses with 16S rDNA sequencing have suggested substantially greater microbial diversity on the ocular surface.[13–15] Shin et al.[15] recently studied the ocular surface by 16S metagenomics techniques in healthy subjects and contact lens wearers. This group reported identification of an average

of 460 operational taxonomic units (OTU) from normal conjunctiva, and a total of 7392 OTUs from among 107 samples. It has been challenging to reconcile these potentially inconsistent results. Our results suggest that application of 16S metagenomics to a paucibacterial sample results in substantial artefactual amplification. Quantitative PCR quantification of 16S amplification suggests the presence of approximately 1 bacterium per 20 recovered epithelial cells, suggesting less than 40 bacteria present per swab (using 5 ng DNA for each sample). It is difficult to reconcile recovery of greater than 100 different genera per sample by this reasoning. That 16S metagenomics analysis is prone to bias and skew has been well recognized.[37–39] The sources for these artifacts include DNA contamination of reagents,[27] errors introduced by PCR amplification, sequencing errors, and misattribution errors.[40] We modeled the potential confound of DNA amplification errors by simulating a PCR amplification containing 20 copies each of four pure 16S sequences (from *S. epidermidis*, *P. acnes*, *Corynebacteria*, and *Streptococcus*) for 25 cycles of amplification using published error rates for different heat-stable
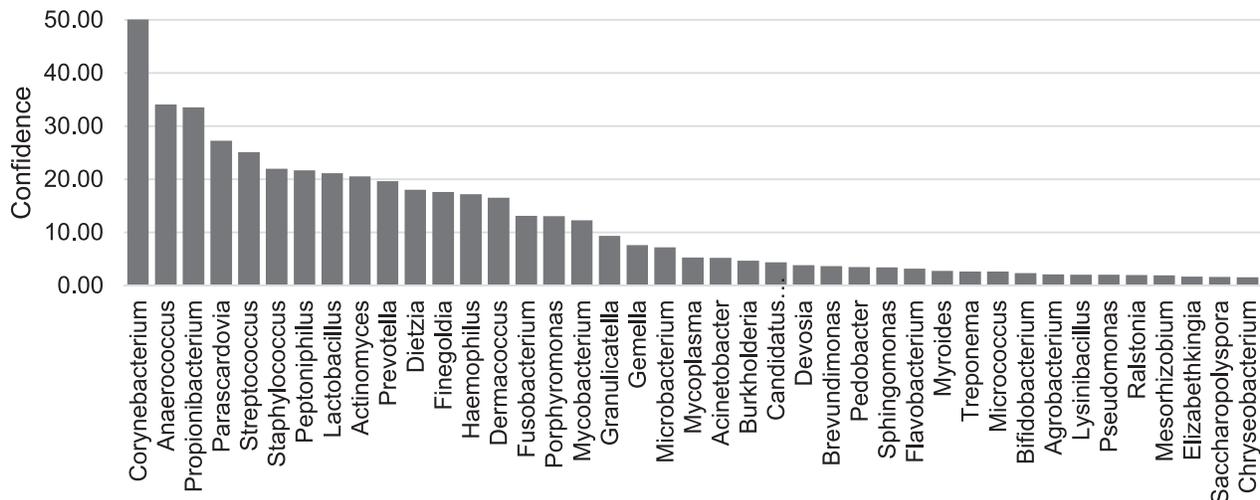
Figure 3. Distribution of confidence heuristic values between conjunctiva and environmental control samples. See text for description of confidence heuristic.
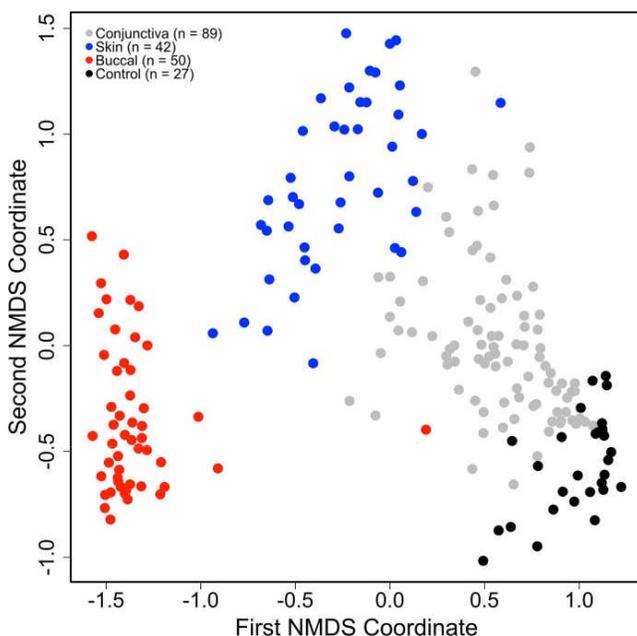
polymerases, ranging from 1×10^{-5} to 1×10^{-7}. Because errors at earlier cycles are propagated, these errors accumulate. For a polymerase with 1×10^{-6} error rate, after 25 cycles of the total 2.68×10^{9} amplicons, 2.92×10^{6} will have incorporated errors (0.11%). This would lead to 36 new genera being identified within the sample. Even with an error rate of 1×10^{-7}, 27 new genera would be identified.

Application of a confidence heuristic combining the magnitude of difference in recovered sequence between conjunctiva and environmental controls as well as the
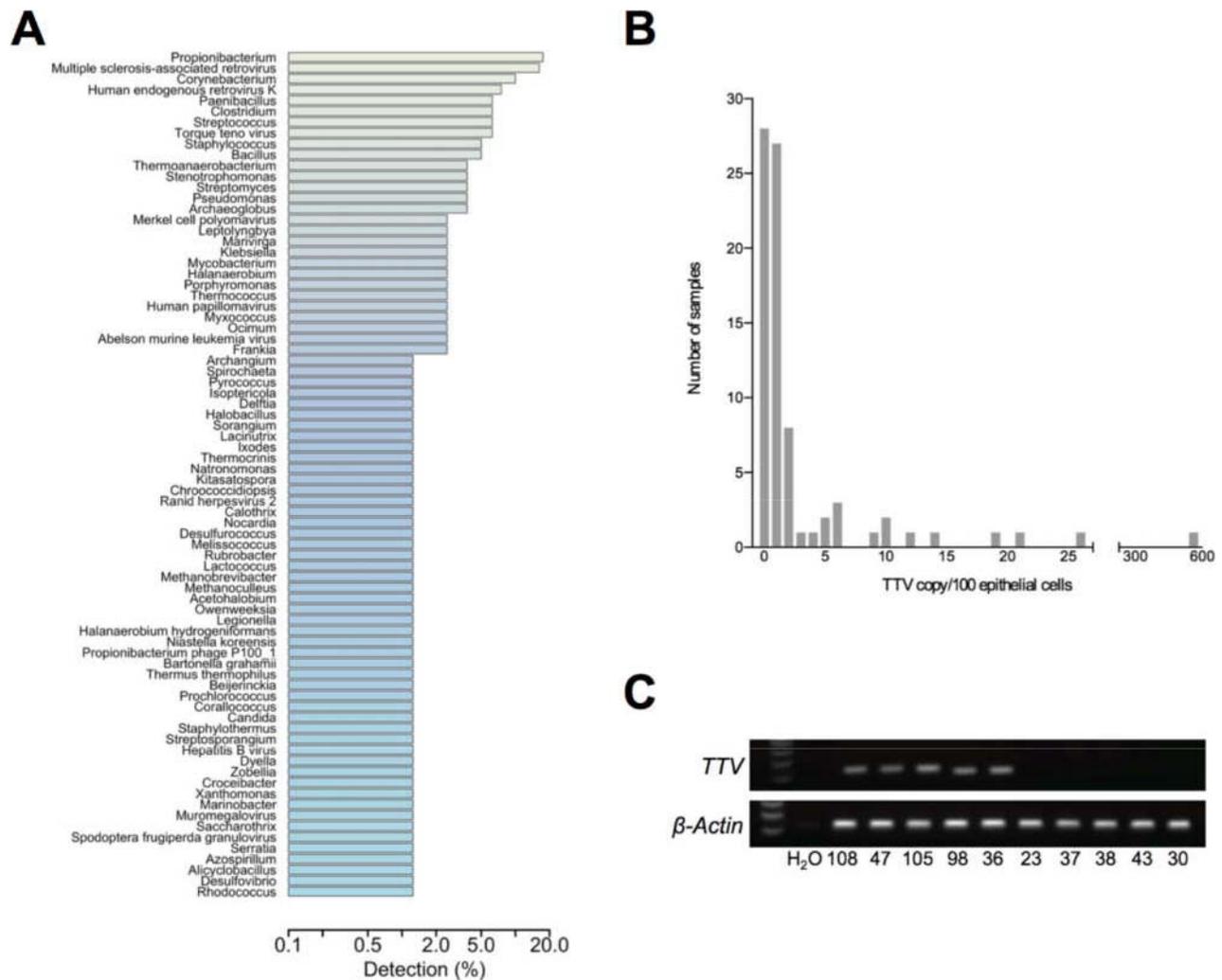

Figure 4. Differences in bacterial community structure, number of genera, and diversity in the various body sites in healthy subjects. Bacterial community structures of the conjunctiva, skin, buccal mucosa, and control samples are represented by NMDS. There were significant differences in the bacterial communities of the healthy conjunctiva and the skin ($R = 0.474$, ANOSIM $P = 0.0001$), healthy conjunctiva and the buccal mucosa ($R = 0.893$, $P = 0.0001$), and healthy conjunctiva and the control samples ($R = 0.536$, $P = 0.0001$).

P value for this difference derived from analysis of several hundred samples produced a distribution ranging over 48 orders of magnitude. Four of the six highest confidence genera were those most frequently recovered by culture and also detected by BRiSK. Conversely, the frequent finding of *Pseudomonas* in 16S data but not culture or BRiSK must be tempered with its markedly lower confidence heuristic. Validation of the presence of specific bacteria not detected by culture will require further studies, which might include performing transcriptomics on ocular surface samples, as this technique analyzes RNA, and hence only detects viable microbes and not DNA contaminants. Similarly, confirmation of suspected microbes by proteomics, immunohistochemistry, or in situ localization would provide more substantial evidence of presence on the ocular surface.

An important finding in this study is that while the ocular surface is not sterile, it is paucibacterial. Our results indicated that there were approximately 2 orders of magnitude fewer bacteria on the ocular surface than there were on the facial skin or the buccal mucosa. The relatively sparse bacterial population of the conjunctiva is likely due to multiple innate antimicrobial properties of tears including antimicrobial peptides, lysozyme, and other innate effectors.[41-44] However, our study also revealed that the ocular surface bacterial community is distinct in comparison with either the buccal mucosa or the skin (Fig. 4). Thus, the conjunctiva is not simply a sparse version of either skin or cheek mucosa. In other niches in the body, modulation of the microbiome can influence health and disease.[45-48] It is conceivable that modulation of the ocular surface microbiome could similarly modulate risk for conditions such as dry eye disease, endophthalmitis, and blebitis, although this hypothesis remains to be tested.

Our most surprising finding was the high prevalence of TTV on the ocular surface of healthy subjects. Of all conjunctiva samples tested, 65% were positive for TTV. This small DNA anellovirus has been correlated with several intraocular inflammatory conditions, specifically SHAPU and culture-negative postoperative endophthalmitis.[10,29] While TTV has been detected previously in tear film,[49] the current study is the first to demonstrate its widespread presence on the ocular surface of a majority of healthy individuals. Torque teno virus induces an adaptive immune response[50] and thus may contribute to ocular surface inflammatory conditions. Future studies will be required to determine the role of this virus in

Figure 5. Detection of bacteria and viruses by BRiSK and quantification of TTV in healthy conjunctiva samples. (**A**) Microbes detected by BRiSK in 80 conjunctiva samples from 80 healthy subjects. (**B**) Distribution histogram of torque teno virus (TTV) copies in 80 healthy conjunctiva samples. (**C**) Torque teno virus–specific PCR products in healthy conjunctiva samples. Five samples on left of plot were TTV-positive and five on right were TTV-negative.

ocular health and disease. While several other viral sequences were identified in a few individuals (i.e., MCV and HPV), these did not appear widespread in this study. It is important to note, however, that none of the techniques used in the current study are able to detect RNA-genome viruses.

The present study has several limitations. The sampled populations included substantial numbers of subjects living in retirement communities, and sampled only one region (urban/suburban Pacific Northwest of United States). For other body sites such as the respiratory tract, specific features of housing conditions such as occupancy have been shown to influence the resident microbiome.[51] It is thus conceivable that other populations—geographic, socioeconomic, or living in different housing—might demonstrate different resident microbial populations. Additionally, the relative health of subjects and their ocular surface was inferred from review of symptoms, history, and external examination. It is possible some subjects had subclinical or asymptomatic disease (such as mild blepharitis) and their ocular surface was therefore not truly healthy. Further studies may examine whether relatively mild ocular surface disease (such as mild dry eye or blepharitis) influences the resident microbiome or virome.

In summary, in the present study has demonstrated that the healthy ocular surface has a unique native microbiome that includes both the viral and bacterial communities. The use of both quantitative and representative sequencing techniques, however, also suggests substantial caution must be applied to interpretation of studies relying solely on 16S metagenomics, as the paucibacterial nature of the conjunctiva appears to induce opportunities for artefactual amplification in this technique. Future studies are needed to confirm the identity, viability, and stability of the microbes identified on the ocular surface, and to determine their relevance to ocular surface disease.

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