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Description of the conjunctival microbiome of normal non-brachycephalic dogs and the effects of antiseptic preparation

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Surgical preparation reduces commensal bacterial load. Currently, no standardized preoperative ocular preparation method in the dog has been reported. Previous studies use culture-based methods to determine commensal bacterial populations. Recent reports suggest that high-throughput sequencing may be superior to culture techniques to determine bacterial communities in the eye and other tissues. The goal of this study was to describe the conjunctival commensal ocular microbiome and bacterial community using DNA sequencing and aerobic cultures of six normal, healthy dogs and investigate the short and long-term effects of an antiseptic protocol on the ocular microbiome. Samples were obtained prior to, immediately following, 24 hours following, and 4 weeks following ocular preparation. The Mississippi State University microbiology laboratory evaluated aerobic cultures, and the Gastrointestinal Laboratory of Texas A&M University performed DNA sequencing. This is the first study to show short and long-term effects of standard ocular surgical preparation on the ocular surface microbiome.

DEDICATION

I would like to dedicate this research to my husband, Chase Seyer, who provides unconditional support and encouragement. I thank him everyday for the love and inspiration especially during the challenging days of my career.

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CHAPTER I

INTRODUCTION

Many surfaces on the body contain a commensal bacterial population. Prior to any surgical procedure, aseptic preparation is recommended to reduce this bacterial load. There are many techniques that have been described to reduce bacterial populations on the eye, however no technique has achieved complete sterility. In addition, a standardized method has not been described in human or veterinary ophthalmology.

Bacterial load is most commonly tested through culture-dependent methods such as aerobic and anaerobic cultures. There is vast literature on commensal bacterial populations on the eye using culture dependent techniques. More recently, technologies have advanced, and culture-independent methods have become more available. This includes next generation sequencing (NGS) methods that help classify bacterial communities. When NGS methods identify microorganisms of the same kingdom, this can be termed the microbiome. Microbiome research has been able to identify previously uncultured microorganisms potentially opening doors to future research questions.

There are minimal ocular studies utilizing these culture-independent methods in dogs. However, the current literature shows similarities among conjunctival samples suggesting a core microbiome in dogs may exist. Manipulations to the ocular surface bacterial population using topical antibiotics have also been investigated through culture-independent samples. Currently, no change in the ocular microbiome in dogs has been documented.

CHAPTER II

LITERATURE REVIEW

Background

The ocular surface is a mucosal interface that harbors a population of commensal organisms. The microbial diversity looking at both the microbiota and microbiome of the ocular surface has been investigated in animals and humans. The microbiota encompasses microbial organisms that belong to all kingdoms. Common microbial kingdoms of the ocular surface include prokaryotes, eukarytoes, protozoa, and fungi. The microbiome is a more focused term which can apply to microbial communities of the same kingdom in similar habitats creating an ecological niche.[1]

In the dog, it has been shown with culture dependent testing that gram-positive bacteria, including *Staphylococcus* spp., *Bacillus* spp., and *Streptococcus* spp., are the most common organisms identified on the ocular surface.[2,3] Similarly, human conjunctiva yields highly gram-positive organisms with the most commonly isolated organism being coagulase-negative staphylococci.[4,5] Since the ocular surface is not a sterile area, surgical preparation is performed to remove commensal bacteria that can be responsible for surgical site infections.[6,7] In veterinary medicine, there is not a described standard surgical preparation for ocular surgery. Variations exist among veterinary ophthalmologists. However, the most commonly used antiseptic in ophthalmic surgery is povidone-iodine due to its safety and efficacy against bacterial and fungal

organisms.[6,8] This antiseptic has been shown via culture-based methods to decrease bacterial colony counts to a level deemed acceptable to prevent surgical site infections.[9,10]

Historically, culture-dependent techniques have exclusively been used to obtain quantitative and qualitative information on the ocular surface bacterial population.[11] These culture-dependent methods may not represent the true population of the ocular surface.[4,12] Positive cultures varied from 29% to 40% in dogs and 16 to 89% in humans when culture-based techniques were employed.[2,4,5,13] Theories for the low yield of organisms with culture-based sampling are the innate mechanisms of the tear film including tear production, eyelid movement, and tear composition, which may keep the ocular surface relatively poorly colonized with bacteria when compared to other sites.[3,14] It has also been suggested that the lack of diversity and amount of bacteria isolated from the ocular surface may be due to the inability to culture and isolate all bacteria that are present within a sample.[12]

Low bacterial yield and diversity of conjunctival culture samples lends the question whether culture-dependent methods are a reliable clinical tool when describing the ocular surface bacterial composition. Culture-independent methods for evaluating the ocular microbiome, such as DNA high-throughput sequencing, have been investigated in cats[15,16], finches[17], koalas[18], and recently in dogs[3,19] and horses[20]. This sequencing method uses 16S ribosomal RNA to better highlight the bacterial community present on the ocular surface[4,21,22] with the goal to establish a core microbiome as a reference for future studies.[3] A recent review highlighted the need for research into the consistencies and alterations in the canine ocular microbiome in order to help understand the role of the ocular microbiome.[23]

Microbiome

Commensal bacterial flora on the ocular surface has been well studied throughout the years in both human and domestic animals. Recently, culture-independent methods have become a popular way to investigate the microbial community of the eye to try to combat the historical limitations of culture-dependent methods. Low culture rates in ocular studies are common in veterinary and human medicine ranging from 20 - 83% of all cultures yielding negative growth.[24–30] Culture-independent methods have been able to identify a much more diverse population of bacteria on the ocular surface of humans suggesting that only a fraction of the true bacterial population is identified with culture-dependent methods.[31]

Advantages of culture-independent studies include increased identification of bacterial populations. In a recent study regarding bronchoalveolar lavage fluid in human patients following lung transplants, culture-independent samples identified a larger percentage of bacterial communities than culture-dependent samples.[32] In addition, culture-independent samples identified bacteria in two samples that were uncultured via culture-dependent methods and clinical improvement was noted after antibiotic therapy proving clinical significance that was initially missed by culture-dependent methods alone.[32] Numerous studies on the human gut microbiome recognize that culture-independent evaluation through next generation sequencing can identify uncultured organisms providing valuable clinical information.[33–37] In addition, it is well known in microbiological research that only a fraction of bacteria are able to be grown with culture-based methods.[31] Next generation sequencing may be able to identify etiologies for disease states in the eye due to the increased identification methods on these samples.

In human and veterinary ophthalmology, there are diseases that an etiology has yet to be confirmed. It has been shown that blepharitis in humans may be caused by a shift of bacteria

residing on the ocular surface.[38] Deviation from the core ocular microbiome of the human eye was found in dry eye patients suggesting that increased bacterial abundance as identified by next generation sequencing may provide more information on the etiology of dry eye disease.[39] Another human study found that the ocular microbiome was distinct between humans with a reactive skin and mucous membrane condition called Stevens Johnson Syndrome, ocular graft vs. host disease, lax eyelid syndrome, and dry eye disease.[40] Research utilizing culture-independent methods is needed in veterinary medicine.

The limitations of microbiome research include the lack of understanding of clinical relevance, determining quality methods of DNA extraction, and limiting technical errors in DNA analysis. However, recent literature such as the Human Microbiome Project has made great strides in the understanding of culture-independent methods and the bacterial populations on the body.[36] Common microbiome profiling technology was initially developed for high bacterial biomass samples. When evaluating the ocular surface microbiome, care should be taken to ensure proper primer sets and decontamination steps are used due to the low microbial biomass recovered from the ocular surface. In regard to dogs, additional research is necessary to understand the importance of this tool and gain more information on the clinical applications of the microbiome.

Leis et al discovered a much more diverse bacterial population in dogs with the most common phyla being Firmicute and Actinobacteria, both gram-positive bacteria.[3] The feline, equine, and canine ocular surface microbiomes have been investigated before and after topical antibiotic administration where a core ocular microbiome was identified in each species.[16,19,20] In horses and dogs, a stable microbial community was identified and shifts in the ocular surface microbiome were not identified regardless of medication application.[19,20] The ocular surface

microbiome in cats was found to change over time; however, medication administration was not associated with this change and it was suggested that environmental factors may play a role on ocular surface microbiome changes in these cats.[16] Research into the canine ocular microbiome is warranted.

Surgical preparation

The goal of surgical preparation is to reduce the commensal bacterial population on the surface of the eye. It has been suggested that infections following ocular surgery are linked to periocular commensal flora.[41–43] Many studies have investigated the most effective disinfectant method prior to ocular surgery to reduce risks of severe ocular infection or endophthalmitis. Surgical site infection rates in human ophthalmic surgery are 0.02-0.2%[29,42,44] and these numbers are even higher when looking at overall bacterial contamination rates with or without leading to endophthalmitis in humans[41,45–51] and dogs[43]. Comparably in dogs, the rate of bacterial endophthalmitis following phacoemulsification is 0.15 -1.4%.[43,52,53] Although the rate of endophthalmitis is low in veterinary and human medicine, this is a tragic complication of intraocular surgery and many times leads to loss of the eye.

In veterinary medicine, the emotional distress of the owner is greatly considered when discussing eye removal procedures, and these owner concerns highlight the impact of surgical complications associated with these procedures. Previous studies have shown that following either bilateral or unilateral enucleation in dogs, 8 - 10% of owners were dissatisfied with their decision and would not consent to the same procedure in the future.[54–56] Full facial expressions and extended eye contact is a key factor in deepening the interspecies bond between humans and dogs.[57–59] This plays a large role in the decision to proceed with enucleation surgery and may compound other hesitations including fear of anesthetic risks, finances, final acceptance of vision

loss, and concern for decreased quality of life following the procedure. Some owners may elect euthanasia over enucleation due to these reasons.[54] The need for diligent ocular preparation prior to surgery is crucial in order to minimize devastating risks that may lead to negative patient outcomes and client satisfaction.

Surgical preparation has evolved over the course of many years in human and veterinary medicine. Differences in methods of surgical preparation exist depending on the type of surgery (clean, clean contaminated, dirty) as well as the anatomic surgical site. In ocular surgery, povidone-iodine is commonly used due to its low rate of side effects when applied topically to the eye.[8,60] Other ocular disinfectants that have been used topically include hypochlorous acid 0.01%, chlorhexidine solution, and liposomal ozone dispersion.[27,28,61] These solutions are not favored due to either decreased efficacy compared to povidone-iodine solutions and/or increased side effects, such as ulceration or irritation, following application. Povidone-iodine has been shown to be efficacious at varying concentrations ranging from 0.5 to 5% in both humans[6,27,30,62–64] and dogs.[43,61,65] Currently, the American Academy of Ophthalmology's guidelines recommend the use of a 5 percent povidone-iodine solution administered to the conjunctival cul-de-sac prior to ocular surgery as appropriate antisepsis.[66] The application method varies greatly in both human and veterinary literature. The efficacy of povidone-iodine as an ocular disinfectant has been investigated using culture-dependent methods. Limited to no research exists investigating the efficacy of ocular antiseptics using culture-independent methods. In addition, no culture-based studies have demonstrated sterilization of the ocular surface following use of povidone-iodine or any antiseptic protocol.[60] The effect of surgical preparation on the ocular microbiome has yet to be investigated in dogs.

Methods of sampling

Sampling of the conjunctiva surface can include impression smears, biopsy samples, or culture-dependent methods.[67] More recently, culture-independent methods have been published investigating DNA sequencing techniques that can be used for conjunctiva. Depending on the reason for sampling, techniques should be atraumatic and high yield. When investigating for bacterial populations, culture-dependent methods have been well described. New literature suggests the use of culture-independent methods may also be useful when investigating bacterial populations on the eye.

Culture dependent methods

Culture-dependent methods have been previously established as a method of sampling the conjunctiva and ocular surface in ophthalmology.[68] Culture-dependent methods have been well established in veterinary ophthalmology to describe bacterial flora on the ocular surface for many years, which is an advantage when utilizing this technique in research and clinical settings. However, culture-based methods may only be able to account for fast-growing microorganisms and microorganisms that can grow on standard media available in a laboratory setting.[69,70]

Ways to obtain a culture-dependent sample of the conjunctiva have been well established. Methods of sampling include the use of a platinum loop, calcium alginate swabs, dry cotton swabs, and wet cotton swabs with calcium alginate swabs having a high reproducibility on bacterial cultures.[68,71,72] Conjunctival cultures can be obtained after application of topical anesthesia[71,73] to improve patient comfort but it is not required for good quality samples.[74] Although topical anesthetics including proparacaine have been shown to have antibacterial effects on the conjunctival flora[75], the use of proparacaine does not affect conjunctival culture results in dogs.[73,76]

Culture independent methods

Culture independent methods have become an important way to evaluate the microbiome of the ocular surface. Next generation sequencing methods have been able to identify a more diverse population of bacteria on the eye.[31,69,70] Commonly, sterile cotton-tipped swabs are utilized in microbiome studies.[23] The technique by which an ocular sample is collected varies in ocular microbiome research. This is a limitation to ocular microbiome studies because the specifics of swabbing techniques including duration of tissue contact, pressure applied during sample collection, and location of ocular surface sampling are crucial for data interpretation.[77] Sterility is imperative due to the high sensitivity in culture-independent research. All samples should be collected and processed in a clean environment to limit environmental contamination. The inclusion of negative controls can be helpful to identify any environmental contaminants.[77] All bacterial 16S rRNA that is present on the sample will be read in the DNA extraction process.[23] Samples should then be processed immediately or stored in a -80 degree freezer until processing.[23]

16S rRNA is a genome sequence commonly used in microbiome research due to its accuracy and is the standard for taxonomic identification.[78–80] By creating a standard for genomic studies, this allows for accumulation of information to create large databases for future research including Silva, Greengenes, and RDP.[79] After sequencing, a taxonomic classification is assigned and entered into a database such as the Greengenes database.[79] The Greengenes database accounts for some of the challenges present in next generation sequencing research such as taxonomic placement of "unclassified" genes, creating consistency in annotation styles, and removing chimeric sequences that can be seen in public databases.[81]

DNA extraction and sequencing

First, the DNA content is extracted from the swab. Previously established techniques have been published regarding DNA extraction methods and commercially available kits following manufacturer's recommendations have allowed for standardized extraction of genomic DNA.[3,23] DNA kits allow for quick, efficient lysing of bacterial cells using detergents to extract DNA with minimal DNA shearing.[82–84] They also are able to produce reproducible results and provide high bacterial diversity.[82] Briefly, chemical and mechanical lysis is accomplished via the use of buffers and centrifuge methods. This extracts and isolates DNA preparing it for PCR amplification. The bacterial 16S rRNA gene region is then amplified. Amplification is commonly performed via high-throughput next-generation sequencing technology such as Illumina MiSeq platform using barcode primers. The sequences are assigned taxonomy by the use of well-established databases as previously mentioned.

Data analysis

After sequencing is complete, the samples are processed and analyzed using software called Quantitative Insights Into Microbial Ecology (QIIME 2). This allows for interpretation of raw data through statistical analysis.[85] The first step of this process is to create the same sequencing depth. Raw sequences are de-multiplexed and filtered by the use of the default parameters for QIIME 2 and a DADA2 plug-in. Chimeric sequences and low quality reads are removed from analysis. Depending on the analysis, amplicon sequence variant (ASV) table can then be created using DADA2. This can identify low abundance ASVs and can be removed from the dataset. In comparison, operational taxonomic units (OTUs) can be used and are assigned and clustered using a protocol in QIIME. If an OTU contains less than a certain percentage similarity to the selected database of the researchers choosing, for example Greengenes, Silva, RDP, it is

removed from the dataset. In addition, sequences that are determined to be non-bacterial, mitochondria, chloroplasts, unassigned, or associated with the phylum cyanobacterium are excluded from the analysis.

Alpha diversity is the diversity within a sample. This must be determined prior to additional analysis in order to compare samples together. Alpha diversity can be established through the use of alpha diversity matrices. These include observed OTUs, Choa1, Shannon index, Simpson index, and observed ASVs. The observed ASVs and Chao1 investigate for species richness. Shannon index and Simpson index investigate for species abundance and evenness. This means that a sample should have a high number of species as well as evenly distributed species within the sample. These index values will be higher with a higher number of species and more even the distribution of these species within a sample. These indices are also calculated in QIIME2. Statistical analysis can then be performed using the software package PRISM. The data must then be tested for normality using, for example, a Shapiro-Wilk test to determine if a post-hoc analysis is required.

Most clinical microbiome data will follow a non-normal distribution; therefore, tests for non-parametric data are used including Wilcoxon matched-pairs signed-ranks test or Kruskal-Wallis analysis of variance (normal distribution but non-parametric). A Wilcoxon matched-pairs signed-ranks test can be used to test for statistical comparison between treatment and control eyes at baseline. A non-parametric Friedman test, followed by a Dunn's multiple comparison post-test can be performed to assess for differences in treatment and control eyes over additional time points.

Beta diversity is the diversity between samples. This is evaluated using a distance matrix. More specifically, weighted and unweighted UniFrac distance matrices are both used to compare

diversity between samples. These UniFrac distance matrices data are visualized by use of principal coordinate analysis plots (PCoA) to investigate for clustering of data points. Clustering of data indicates homogeneity between the samples. Lack of clustering indicates a more heterogeneous population between data. Furthermore, unweighted and weighted UniFrac distance matrices investigate different aspects of the data. The unweighted UniFrac distance matrix is a qualitative measure that includes the species that are present or absent based on phylogenic distances. For example, if there are two species from a different phylum, they will be further in distance. The weighted UniFrac distance matrix is a quantitative measure that investigates the abundance of species. Assessment of differences in bacterial composition between samples is then analyzed statistically using an Analysis of Similarity Test (ANOSIM). A sample statistic or R-value closer to 1 indicates a very high difference within the composition of the samples proceeding down to 0 that indicates that the samples were very similar and the composition was not different between samples.

CHAPTER III

DESCRIPTION OF NON-BRACHYCEPHALIC CANINE CONJUNCTIVAL MICROBIOME BEFORE AND AFTER APPLICATION OF AN ANTISEPTIC PREPARATION

Introduction

The goal of surgical preparation is to reduce commensal bacterial load in order to prevent surgical site infections and postoperative complications such as bacterial endophthalmitis.[6,60,86–89] Surgical preparation practices in veterinary medicine are derived from human protocols identifying reductions in commensal bacteria following use of common antiseptics including b chlorhexidine gluconate, povidone-iodine, and hexachlorophene.[90–94] These protocols have demonstrated similar reductions in bacterial load when used in veterinary medicine utilizing chlorhexidine gluconate, chlorhexidine diacetate, and povidone-iodine antiseptics.[9,95–97]

In both human and veterinary medicine, the specifics of ocular preparation are not standardized. Currently, literature demonstrating consistent sterilization of the ocular surface does not exist. The American Academy of Ophthalmology has published guidelines to attempt to standardize aspects of ocular surgical preparation by recommending use of a povidone-iodine 5% solution applied to the conjunctival cul-de-sac with a contact time of 3 minutes.[60,98,99] This varies slightly from the guidelines of the European Society of Cataract and Refractive Surgeons that suggest povidone-iodine solutions of 5 – 10% applied for at least 3 minutes.[100] There are no standardized guidelines for ocular preparation in veterinary medicine.

Most antiseptic studies utilize culture-dependent methods to isolate and identify reductions in bacterial organisms. Although the ocular surface has a low bacterial biomass, enumeration and identification of bacteria has been established in ocular bacteriology by utilization of cotton and/or alginate swabs on culture mediums in both human and veterinary studies.[68] There are limitations noted with culture-based techniques with a bias toward less fastidious organisms that are easily grown in the laboratory. Recent literature may suggest that high-throughput sequencing may be superior when determining the bacterial community of the ocular surface. Currently, the effect of a standard ocular preparation of the canine ocular microbiome is unknown.

Many studies of the human ocular microbiome utilizing culture independent methods have been conducted to expand upon the knowledge of microbial communities of the ocular surface. The ocular surface microbiome has been shown to change in contact lens wearers, dry eye patients, and patients with bacterial infections.[40,101–103] Culture-based and culture-independent methods are not equivocal in their sampling specificity and sensitivity and clinical relevance of culture-independent methods is unknown at this time. In veterinary medicine, minimal studies exist on the ocular surface microbiome and have not identified shifts in microbial populations following use of topical antibacterial medications.[16,104]

The aims of this study were to describe and compare the commensal conjunctival microbiome of non-brachycephalic healthy dogs living in Mississippi using aerobic cultures and DNA high-throughput sequencing, and to investigate the effects of a standard antiseptic preparation on the conjunctival microbiome immediately following, 24 hours, and 4 weeks following ocular preparation with 1:50 povidone-iodine solution. It was hypothesized that a common commensal ocular microbiome would be identified and would be more diverse than the

bacterial population recovered by standard culture-based techniques. In addition, the ocular surface microbiome would change following ocular preparation.

Materials and Methods

Participants

Staff at Mississippi State University College of Veterinary Medicine was surveyed to identify six non-brachycephalic dogs for the study. Dogs were identified as possible participants based on health status, skull conformation (non-brachycephalic), and were required to be free of ophthalmic disease. All dogs were required to have a normal physical examination and owners were required to complete a health survey confirming they were free of underlying systemic disease. After the health status was confirmed, a full ophthalmic examination including Schirmer tear test I (Merck Animal Health, Summit, NJ, USA), fluorescein stain (Fluoro-I-Strip; AT; Ayerst Laboratories, ST. Laurent, Quebec, Canada), rebound tonometry (TonoVet; Icare Finland, Espoo, Finalnd), slit lamp biomicroscopy (Kowa portable slit-lamp SL-17; Kowa Optics), and non-dilated indirect ophthalmoscopy (Vantage Plus Digital LED; Keeler Ophthalmic Instruments, Broomall, PA, USA) with a 28 diopter condensing lens (Volk Optical; Mentor, OH, USA) was performed. When six healthy non-brachycephalic dogs free of ocular surface disease were identified, these dogs were enrolled in the study. A client consent form was completed prior to the study. The Mississippi State University Institutional Animal Care and Use Committee approved this study.

Antiseptic Preparation

A standard ocular antiseptic preparation was applied to one eye. The fellow eye did not receive an ocular antiseptic preparation but was sampled at each time point to serve as a control.

A random number generator determined the control eye. Briefly, 5 milliliters of 1:50 povidone-iodine solution was administered over the corneal and conjunctival surfaces slowly in the treatment eye, then 10 cotton-tipped applicators soaked in 5 milliliters of 1:50 povidone-iodine solution and 5 cotton-tipped applicators soaked in 5 milliliters of sterile saline were passed around the superior and inferior conjunctival fornices. This preparation was performed in a sterile manner using sterile gloves, applicators, and preparation basins. Conjunctival swabs were collected at baseline (both eyes), immediately following preparation (treatment eye only), 24 hours post-preparation (both eyes), and 4 weeks post-preparation (both eyes). An aerobic culture sample and an Isohelix DNA sample were obtained from left and right eyes. No ocular preparation was applied prior to the 24 hour or 4 week time point.

Conjunctival Sample Collection

Two drops of proparacaine hydrochloride 0.5% ophthalmic solution were administered into each eye prior to obtaining any swab samples. Two drops of proparacaine were placed on unused Isohelix swab (Isohelix; Midsci, St. Louis, MO) and culture swab (Starswab II; Etobicoke, Ontario, Canada) at the same time and place of subject testing to serve as negative controls to confirm lack of environmental contamination. A coin toss determined which eye was sampled first and the order of culture and Isohelix swab sample collection. The same order was used for each subsequent sampling time. The process for sample collection proceeded as follows; culture and Isohelix swabs were introduced to the inferior conjunctival fornix and rolled from the medial to lateral aspect ten times with static pressure; the Isohelix swabs were placed in a Powerbead tube (Qiagen; Hilden, Germany) and culture swabs were returned to aerobic culture medium.

The aerobic culture samples were immediately processed at the Mississippi State University College of Veterinary Medicine Diagnostic Laboratory System. Isohelix swabs in the PowerBead tubes were stored in a -80°C freezer until all study samples were obtained and then were shipped overnight to the Gastrointestinal Laboratory at Texas A&M University College of Veterinary Medicine on dry ice.

DNA Extraction and Sequencing

Illumina sequencing of the bacterial 16S rRNA genes were performed using primers 515F (5'-GTGYCAGCMGCCGCGGTAA)[105] to 806RB (5'-GGACTACNVGGGTWTCTAAT)[106] at the MR DNA laboratory (Shallowater, TX).

Sequences were processed and analyzed using a Quantitative Insights Into Microbial Ecology 2 (QIIME 2)[107] v 2018.6 pipeline. Briefly, the sequences were demultiplexed and the ASV table was created using DADA2.[108] Prior to downstream analysis, sequences assigned as chloroplast, mitochondria, and low abundance ASVs, containing less than 0.01% of the total reads in the dataset were removed.

Prevalence-based filtering of putative contaminant ASVs was performed using the R package decontam (v0.99.1).[109] A DNA extraction blank contemporaneously generated and processed in parallel with biological samples was used as negative control in the filtering procedure. ASV tables were used as the input for the isContaminant() function (pss, method = "prevalence", neg = "is.neg", threshold = 0.5). The table with the contaminants generated was visualized with ggplot2, and contaminants were then filtered from the ASV table for downstream analysis. All samples were then rarefied to even sequencing depth, based on the lowest read depth of samples, to 646 sequences per sample.

Alpha diversity was measured with the Chao1 (richness), Shannon diversity, and observed ASVs metrics within QIIME2. Beta diversity was evaluated with the weighted and unweighted phylogeny-based UniFrac[110] distance metric and visualized using Principal Coordinate Analysis (PCoA) plots, generated within QIIME2.

Statistical Analysis

ANOSIM (Analysis of Similarity) test within PRIMER 7 software package (PRIMER-E Ltd., Luton, UK) were used to analyze significant differences in microbial communities between groups/time points. All datasets were tested for normality using Shapiro-Wilk test (JMP Pro 11, SAS software Inc.). A 2-way RM ANOVA test was performed (Prism v.9, Graphpad Software Inc.) and adjusted for multiple comparison using Benjamini and Hochberg's False Discovery Rate[111] at each taxonomic level and a P value < 0.05 was considered statistically significant. Post hoc Sidak's multiple comparison test was used to determine the group differences in bacterial taxa at each time point. The bacterial diversity between culture-based samples and DNA sequencing samples were compared. A qualitative comparison was made between culture-based swabs and DNA sequencing on bacterial yield.

Sample sizes calculations were conducted using G*Power 3.1.9.2 software[28] to determine the number of dogs required to detect a difference in the number of species detected by PCR and culture through analysis by paired t-test. The assumptions used for the calculations included an alpha level of 0.05, a power of 0.80, a correlation between the two groups of 0.5, a two-tailed test, and a standard deviation of 2.32 (the greatest standard deviation found in the preliminary culture results) for either method. The analysis indicated six dogs would be sufficient to detect a difference as small as 3.5 species.

Culture-based evaluations of samples were performed to investigate the changes of ocular preparation on the cultured flora of the canine conjunctiva. The difference between mean numbers of isolates cultured from the same eye of each dog prior to and immediately after treatment was assessed by a paired t-test. The effect of treatment, sample time, and their interaction on the number of isolates cultured was assessed by a linear mixed model, accounting for sampling both eyes of each dog over multiple sample times.

Results

Culture-dependent Samples

Predominantly gram-positive bacteria grew from aerobic cultures in all six dogs. Aerobic cultures identified a total of eleven bacterial families from the entire study. At baseline, a total of six bacterial families were identified including Bacillaceae (35%), Staphylococcaceae (30%), Streptococcaceae (20%), Moraxellaceae (5%), Micrococcaceae (5%), and Neisseriaceae (5%). One dog was noted to have growth of *Bacillus cereus* in both the control and treatment eye at all time points in the study. Nineteen percent (8/42) of all cultures throughout the study had no growth and of these samples four were from control eyes and four were from eyes that received the ocular preparation. For positive cultures, total number of bacterial isolates grown per sample varied from 1-6 isolates (Table 1) and total number of bacterial isolates grown per dog varied from 4 – 23 isolates (Table 2).

Table 1 Bacterial isolates per sample

Number of bacterial isolates grown per sample	Number of samples from control group	Number of samples from treatment group	Total number of samples
1	5	7	11
2	4	9	13
3	3	2	5
4	1	2	3
6	1	0	1

Table 1 demonstrates the total number of bacterial isolates grown per sample on aerobic cultures.

Table 2 Bacterial isolates per dog

Number of bacterial isolates grown per dog	Number of dogs
23	1
13	3
11	3
4	3
10	2

Table 2 demonstrates the total number of bacterial isolates grown per dog on aerobic cultures.

No significant difference was detected between the mean number of isolates cultured immediately before and after treatment ($p=0.465$) with culture-based methods. Bacterial isolate growth was decreased in four out of six dogs (66.6%) immediately following application of topical antisepsis. Bacterial isolate growth increased from one to four isolates post-treatment in one dog, and remained stable in one dog with *Bacillus cereus* growth at all time points. No significant effect of treatment, sample time, or their interaction was detected on mean number of isolates cultured ($p>0.393$).

Culture-independent Samples

Sequence Analysis

A total of 43 samples were sequenced including one negative control. Forty-two samples were collected from twelve eyes at 4 time points and yielded good quality sequencing. ASV representation as demonstrated by Good's coverage index and rarefaction analysis in each sample had either reached or trended towards a plateau indicating adequate sequencing. There were a total of 1,167,937 sequences amplified (647 - 135,053 sequences per sample) and rarified to 646 sequences per sample. Each sample was then investigated for abundance of bacteria.

Dogs at Baseline

Species richness and diversity at baseline

Alpha diversity metrics were used to investigate richness within a sample. Three diversity metrics were used including observed OTUs, Chao1, and Shannon index. No statistically significant difference was noted between eyes at baseline by use these three alpha diversity metrics.

Microbial community structure at baseline

Beta diversity distance matrices were used to investigate richness between samples. Treatment and control eyes at baseline did not have a statistically significant difference in their community diversity as show by the weighted UniFrac ($R=-0.15$, $p>0.05$) and unweighted UniFrac ($R=-0.156$, $p>0.05$) distance matrices. In addition, there was a lack of clustering observed on principal coordinate analysis plots (PCoA) for weighted and unweighted UniFrac distance matrices which suggests community structure between control and treated eyes did not differ.

Microbial community composition at baseline

Bacterial taxa relative abundance at baseline was not statistically different between treatment and control eyes using a 2-way RM ANOVA. The bacterial composition of the ocular surface was described by averaging samples from all 6 dogs enrolled in the study. Thirteen bacterial phyla were identified with the most predominant phyla being Proteobacteria (43.6%), followed by Actinobacteria (35.5%), Bacteroidetes (8.3%), Firmicutes (7.2%), and Tenericutes (2.3%). Of these, only two phyla, Proteobacteria and Actinobacteria, were present in all eyes.

A sum of 64 bacterial families were identified. Only four families were present in at least 10/12 eyes including Moraxellaceae (11/12 eyes, Sphingomonadaceae (11/12 eyes), Corynebacteriaceae (10/12 eyes), and Comamonadaceae (10/12 eyes). The most frequently identified families included Corynebacteriaceae (27.9%), Neisseriaceae (10.8%), Pasteurellaceae (7.2%), Porphyromonadaceae (4.2%), and Sphingomonadaceae (3.0%). Micrococcaceae (2.7%), Bacillaceae (2.1%), Streptococcaceae (0.4%), and Staphlyococcaceae (0.2%) represented a low relative abundance of bacterial taxa identified via culture-independent methods in the current study.

Baseline aerobic cultures in the current study included Bacillaceae, Streptococcaceae, Staphlyococcaceae, Moraxellaceae, Micrococcaceae, and Neisseriaceae. When comparing the families identified with bacterial cultures versus DNA sequencing samples, there were differences in the relative abundance of each family identified. Moraxellaceae, Micrococcaceae, and Neisseriaceae each comprised 5% of all cultured bacterial families with aerobic cultures. In comparison, the relative abundance of these bacterial families identified with culture-independent methods represented 1.9%, 2.7%, and 10.8% of all sequenced bacterial families,

respectively. Although overall bacterial composition remained constant between eyes and dogs there was minor variation in bacterial taxa.

Control eyes over time

Species richness and diversity over time

Alpha diversity metrics indicated there was no statistically significant difference noted between control eyes over time.

Microbial community structure over time

Beta diversity distance matrices were used to investigate richness between samples for control eyes over time. Controls eyes did not significantly change in regards to their diversity structure over time as show by the weighted UniFrac distance matrices (R=-0.13, R=-0.069, R=-0.057 for time points baseline vs 24 hours, baseline vs 4 weeks, and 24hr vs 4 weeks, respectively, $p>0.05$) and unweighted UniFrac distance matrices (R=-0.072, R=-0.017, R=-0.106 for time points baseline vs 24 hours, baseline vs 4 weeks, and 24hr vs 4 weeks, respectively, $p>0.05$). No clustering on PCoA was noted for weighted and unweighted UniFrac distance matrices.

Microbial community composition over time

A 2-way RM ANOVA test was performed to investigate for any change in abundance of bacterial taxa in control eyes over time. Variation in bacterial taxa was noted and shown in figure 1. No statistically significant change was noted in relative abundance in bacterial taxa between or within control eyes overtime.

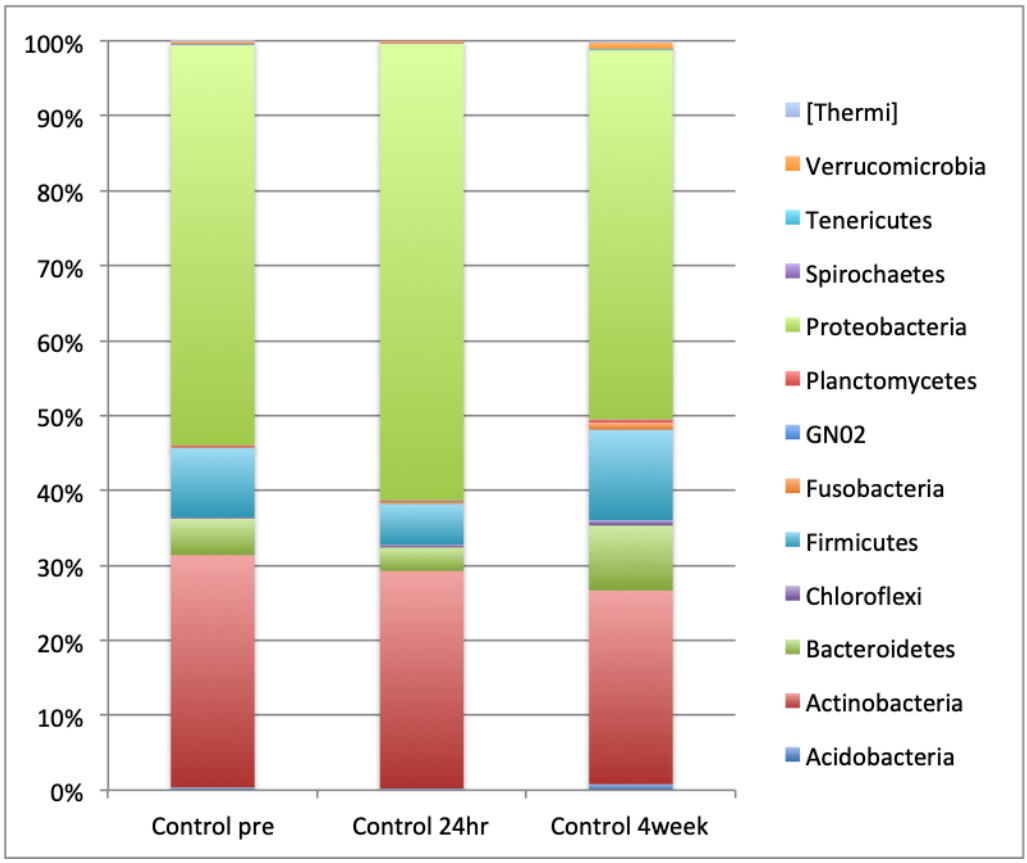

Figure 1 Microbial composition over time in control eyes at phylum level

Experimental eyes after antiseptic preparation

Species richness and diversity after antiseptic preparation

Alpha diversity metrics indicated there was no statistically significant difference noted between treated eyes at any time point.

Microbial community structure after antiseptic preparation

Treated eyes did not significantly change their diversity composition over time. There were no statistically significant changes in diversity composition on weighted UniFrac distance matrices (R=-0.07, R=-0.098, R=-0.061, R=-0.07, R=-0.065, R=-0.093 for time points: baseline

vs immediately following treatment, baseline vs 24 hours, baseline vs 4 weeks, immediately post treatment vs 24 hours, immediately post treatment vs 4 weeks, 24hrs vs 4 weeks, respectively, $p>0.05$). This suggests that the abundance of bacteria were not significantly altered with the ocular treatment used in this study.

There was a statistically significant difference in diversity composition between treatment eyes immediately following treatment when compared to treated eyes 4 weeks post treatment on unweighted UniFrac distance matrices ($R=0.263$, $p<0.05$). This suggests that the diversity composition was changed in terms of presence and absence of bacteria following the treatment used in this study after a 4-week period of time. No further differences in diversity composition were noted at any other time point in this study based on unweighted UniFrac distance matrices ($R=-0.046$, $R=-0.076$, $R=0.063$, $R=-0.024$, $R=0.017$ for time points baseline vs immediately following treatment, baseline vs 24 hours, baseline vs 4 weeks, immediately post treatment vs 24 hours, 24hrs vs 4 weeks, respectively, $p>0.05$). This is supported by the unweighted UniFrac PCoA plots which demonstrated slight clustering immediately following treatment which normalized and began to cluster closer to baseline at the 4-week time point. No further clustering pattern was noted for any other time point on the unweighted UniFrac PCoA plots. Although mild clustering was noted, this was not statistically significant as demonstrated by the sample statistic ($R=-0.021$, $p>0.05$).

When evaluating UniFrac distances between control and treated eyes, there were no statistically significant differences in bacterial communities noted between groups. This suggests that the bacterial diversity identified in control eyes and treatment eyes was not determined or altered by the treatment used in this study.

Microbial community composition after antiseptic preparation

A 2-way RM ANOVA test was performed to investigate for any change in abundance of bacterial taxa in eyes that received an ocular preparation with iodine solution. Figures 2 show variation in bacterial taxa. No statistically significant change was noted in relative abundance in bacterial taxa between or within treatment eyes over time.

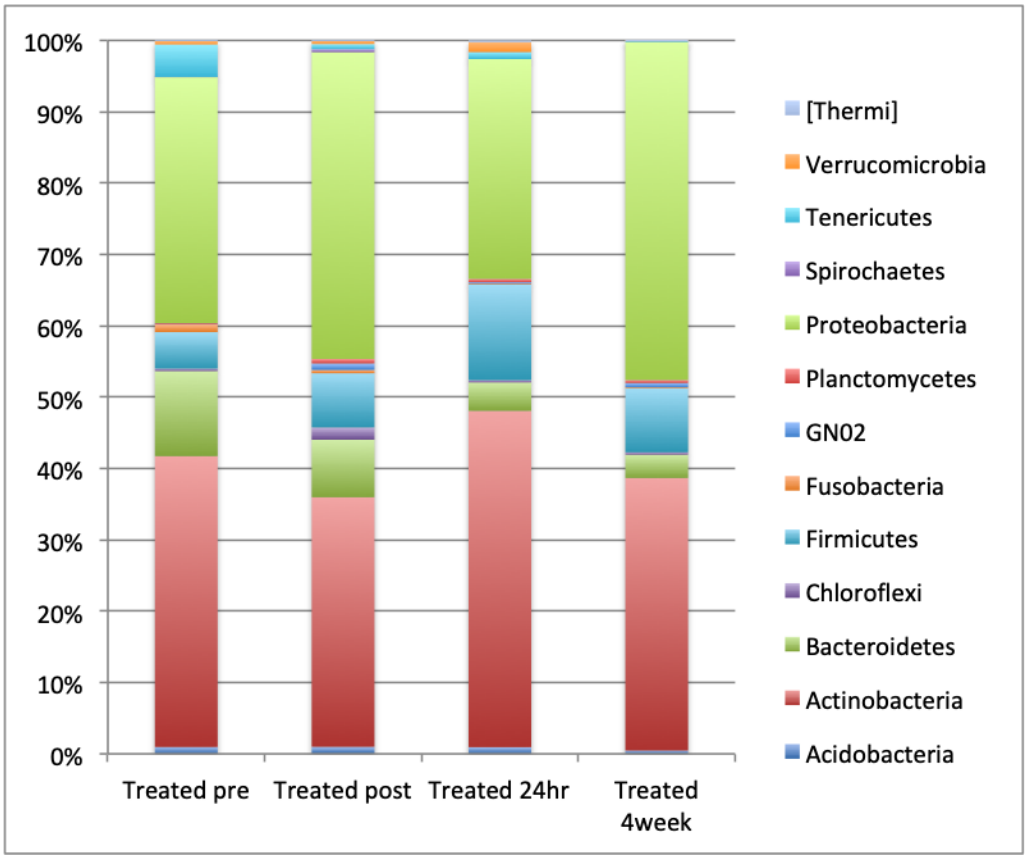

Figure 2 Microbial composition over time after antiseptic preparation at phylum level

Discussion

It was identified that the conjunctival surface microbiome identified by culture dependent and independent methods remains stable over time with and without the use of a topical ocular

preparation. DNA high-throughput sequencing identified a more diverse bacterial population than corneal aerobic cultures. Bacterial families identified were similar between the two techniques.

Conjunctival aerobic cultures in the current study yielded a comparable bacterial growth rate compared to previous literature with a positive bacterial growth in 79% of all aerobic cultures with at least 1 species isolated. The conjunctival surface yields a low microbial biomass via culture-dependent methods and positive bacterial culture rates range from 20-80%. [24–30,63,112–114] The positive culture rate in the current study was 81% with 19% of all aerobic conjunctival cultures yielding no bacterial growth. Of the samples with negative growth, 50% were from the control group. Corneal aerobic cultures revealed a baseline population of Bacillus, Staphylococcus, and Streptococcus spp. These findings are consistent with previously published studies identifying Bacillus, Staphylococcus, and Streptococcus as some of the most commonly isolated organisms in healthy dog eyes via aerobic cultures.[24,25]

The baseline bacterial phyla identified in healthy dogs in this study before an antiseptic ocular preparation included Proteobacteria, Actinobacteria, Bacteroidetes, Firmicutes, and Tenericutes. These bacterial phyla were present in all eyes at baseline. Current literature suggests that this population of bacteria may resemble a core microbiome in dogs. Leis et al found that conjunctival flora of healthy dogs located in Saskatchewan, Canada consisted of the same phyla found at baseline in the current study.[104] In a study investigating healthy dogs in Texas, the conjunctival flora was found to be predominated by Proteobacteria, Actinobacteria, Bacteroidetes, Firmicutes, and Fusobacteria.[19] Similarly, Banks et al identified that conjunctival samples from healthy dogs in Missouri consisted of the same core microbiome of Proteobacteria,

Actinobacteria, Bacteroidetes, and Firmicutes.[115] In the current study, Moraxellaceae was also commonly identified in many samples.[115]

Bacterial composition of the conjunctival surface identified differed between culture dependent and independent methods in the current study. Conjunctival aerobic cultures identified 11 bacterial families while DNA high-throughput sequencing identified 64 bacterial families. Corneal aerobic cultures identified 1 - 4 bacterial species while DNA high-throughput sequencing revealed 75 species. These findings suggest that next generation sequencing (NGS) samples were rich in diversity and composition.

Bacterial composition did not significantly change in control or treated eyes of healthy dogs without ocular disease after the antiseptic ocular preparation used in this study with conjunctival aerobic cultures or DNA high-throughput sequencing. However, clustering of data points was seen immediately following ocular preparation and a return to the baseline pattern was noted by four weeks following preparation. Overall this finding was not significant; however, it parallels the findings from aerobic cultures demonstrating reduction in bacterial isolate growth immediately following antiseptic ocular preparation in 66% of dogs. In a previous study investigating bacterial isolates of the conjunctival sac of dogs, it was found that 39% of clinically normal dogs had positive cultures whereas 100% of dogs with ulcerative keratitis had positive cultures.[25] Dogs with ophthalmic disease carry a higher bacterial load; therefore, a statistically significant shift in bacterial communities following topical antisepsis may be more evident in these dogs. The small sample size is a limitation of the current study. It is suggested that increasing the sample size may allow for identification of statistical significance when working with aerobic samples that yield low percentages of data.

Health care professionals owned all dogs enrolled in the current study. Human and veterinary medicine have identified the increased prevalence of commensal methicillin-resistant *Staphylococcus* among healthcare workers.[116–120] Susceptibility profiles were not completed on the bacteria in the current study. However, one dog was noted to have stable bacterial isolate growth throughout the study following antiseptic preparation with *Bacillus cereus* being isolated at all time points in both eyes. Bacillus cereus has been identified to cause clinical infections in humans[121] and is a common commensal bacteria of hair, skin, and conjunctiva in dogs.[24,122] Because a veterinarian owned this dog, further investigation into the susceptibility profile of the *Bacillus cereus* identified in this dog would be indicated.

The ocular preparation used in this study was derived from a similar protocol used prior to ophthalmic surgery in the authors' current practice. In a study by Roberts et al, the antibacterial action of dilute povidone-iodine solutions at different concentrations demonstrated absence of bacteria on the ocular surface following all dilutions measured by aerobic culture.[65] In the current study, the haired skin was not clipped or prepared, which differed from Roberts et al, in which the periocular hair was clipped and an aggressive periocular scrub was completed. Antisepsis in human cataract patients many times includes a combination of scrubbing of the periorbita, lashes, flushing of the fornices, and/or placement of antiseptic soaked gauze prior to surgery.[29,30,63,113,123] Isolates on the periorbital skin have been found to be similar to those found in the conjunctival sac of dogs.[115] The authors suspect that preparation of the periocular surfaces may play a role in decreasing bacterial isolates post-preparation. In addition, variations in ocular preparation can have large impacts on the reduction of bacterial load.

Contact time of povidone-iodine has been studied in human and veterinary literature.[124] Diluted povidone-iodine solutions have bactericidal activity following a minimum contact time

of 10 – 30 seconds.[64,93,124,125] In general, 3 to 5 minute contact time is recommended for appropriate antisepsis.[60,98,99,126] In the current study, the antiseptic preparation was standardized to 5 minutes for all dogs. This included an estimated three minutes of contact time of povidone-iodine 5% solution followed by 2 minutes of sterile saline contact time.

Conclusion

The current study identified a baseline microbiome in healthy dogs living in Mississippi. Clinical relevance of the ocular microbiome is unknown at this time. DNA high-throughput sequencing has a higher bacterial yield than aerobic cultures. It is possible that bacteria recovered from DNA high-throughput sequencing may be linked to ocular disease in dogs. This study provides information regarding the conjunctival microbiome following ocular antiseptic preparation with a dilute povidone-iodine solution.

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