Oregon Health & Science University
School of Medicine

Scholarly Projects Final Report

Title *(Must match poster title; include key words in the title to improve electronic search capabilities.)*

Development and Characterization of a Method for Assessing Microbial Contamination Rates of Multi-Use Ophthalmic Solutions

Student Investigator's Name

Nina Kostur

Date of Submission *(mm/dd/Yiyi)*

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Graduation Year

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Project Course *(Indicate whether the project was conducted in the Scholarly Projects Curriculum; Physician Scientist Experience; Combined Degree Program [MD/MPH, MD/PhD]; or another course.)*

Scholarly Projects Curriculum

Co-Investigators *(Names, departments; institution if not OHSU)*

Aiyin Chen, MD (Casey Eye Institute), William B. Messer, MD, PhD (Dept. Internal Medicine, Division of Infectious Disease), Felicity J. Coulter, BS, MS (Dept. of Microbiology and Immunology), Courtney Michelet, BS (Dept. of Microbiology and Immunology)

Mentor's Name

Aiyin Chen, MD and William Messer, MD, PhD

Mentor's Department

Ophthalmology and Infectious Disease

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Concentration Lead's Name

Alex Foster, MD, MPH

Project/Research Question

To develop a method for evaluating and comparing the microbial contamination arising use of multi-use eye drops and to compare rates of contamination among samples from 2 different locations on the eye-drop bottles, thereby contributing to the data supporting or refuting the validity the current guideline for multi-use ophthalmic solutions which specifies a 1 month in-use expiry date.

Type of Project *(Best description of your project, e.g., research study, quality improvement project, engineering project, etc.)*

Research Study

Key words *(4-10 words describing key aspects of your project)*

Multi-use eye drops, microbial contamination, ocular infection, infection prevention, ophthalmic medications

Meeting Presentations

If your project was presented at a meeting besides the OHSU Capstone, please provide the meeting(s) name, location, date, and presentation format below (poster vs. podium presentation or other).

American Glaucoma Society Annual Meeting. Austin, TX. 3/2/2023. Poster presentation

Publications *(Abstract, article, other)*

If your project was published, please provide reference(s) below in JAMA style.

Submission to Archive

Final reports will be archived in a central library to benefit other students and colleagues. Describe any restrictions below (e.g., hold until publication of article on a specific date).

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Next Steps

What are possible next steps that would build upon the results of this project? Could any data or tools resulting from the project have the potential to be used to answer new research questions by future medical students?

This project outlines a method for quantifying contamination rates of multi-use eye drops in the outpatient setting. The number of drops sampled, however was small. This method can be used to determine the incidence contamination for a larger sample size, among other medication types, and at different time points.

Please follow the link below and complete the archival process for your Project in addition to submitting your final report.

https://ohsu.ca1.qualtrics.com/jfe/form/SV_3ls2z8V0goKiHZP

Student's Signature/Date *(Electronic signatures on this form are acceptable.)*
This report describes work that I conducted in the Scholarly Projects Curriculum or alternative academic program at the OHSU School of Medicine. By typing my signature below, I attest to its authenticity and originality and agree to submit it to the Archive.

X

Student's full name

Mentor Name

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Report: *Information in the report should be consistent with the poster but could include additional material. Insert text in the following sections targeting 1500-3000 words overall; include key figures and tables. Use Calibri 11-point font, single spaced and 1-inch margin; follow JAMA style conventions as detailed in the full instructions.*

Introduction *(≥250 words)*

Multi-use eye drops are repeatedly handled and shared among patients and thus carry a risk of transmitting both opportunistic and pathogenic microorganisms.[1-3] Contaminants primarily consist of commensal organisms that belong to environmental and human flora, though pathogens such as *Pseudomonas aeruginosa, Serratia marcescens, Proteus mirabilis,* and *Staphylococcus aureus* can be present.[1-5]. Though multi-use solutions contain preservatives designed to eliminate microorganisms and contaminated eye drops are an infrequent cause of ocular infection, there have been reports of conjunctivitis, keratitis, and even endophthalmitis resulting from contaminated eye drops.[6-9]

The incidence of microbial contamination of eye dropper bottles varies widely in the literature, ranging from 0.07% to 70%. On the whole, antibiotics and those with preservatives appear to have lower contamination rates compared to other kinds of ophthalmic solutions such as glaucoma medications and those without preservatives.[5,10] Location of sampling (drops vs. residual fluid vs. cap/tip) may play a role in rates of contamination, though there is no consensus as to which location is most likely to be contaminated. While some studies suggest that the tip was the most frequently contaminated, others report that the drops and the residual fluid were more likely to be contaminated.[3,4,11-13] Overall, there is a lack of agreement in the literature and the true incidence of microbial contamination of multi-use vials remains unknown.

Despite this uncertainty, the US Pharmacopeia standard 797 (USP 797) provides guidelines for the use of sterile preparations and multidose vials. These policies have been adopted by the Joint Commission of the American Journal of Ophthalmology, and thus, they are the standard practice in the field of ophthalmology.[14,15] The USP 797 states: "multiple-dose containers (e.g., vials) are formulated for removal of portions on multiple occasions because they contain antimicrobial preservatives. The beyond-use date after initially entering or opening (e.g., needle-punctured) multiple-dose containers is 28 days unless otherwise specified by the manufacturer. If the vial is labeled as a multi-dose vial or container, then the dating should not exceed 28 days UNLESS the manufacturer has data to support longer dating." [14,16]

While the use of contaminated eyedrops and their containers may give rise to serious ocular infections, more nuanced guidelines may be beneficial in reducing the amount of waste associated with eye drop usage. Waste, both in the monetary and the material sense, is an issue that continues to plague medicine and is one of the many factors contributing to exorbitant costs within healthcare and an ever-expanding pollution problem for our world.[17,18] The healthcare sector and upstream activities accounted for 16% of US GDP and contributed an estimated 9-10% of total US carbon emissions in 2013. Production of greenhouse gases may account for up to 123,000 to 381,000 disability-adjusted life-years in future health damages which result from increases prevalence of extreme weather and flooding, which reduce food production capability and subsequent malnutrition, vector-borne disease, respiratory disease, and other effects.[17,19]

Ophthalmology is by no means exempt from this issue. A 2019 study published in JAMA Ophthalmology examining medication waste in cataract surgery found that unused product accounted for an estimated $195,200 and a potential environmental effect of 2498 kg of carbon dioxide equivalents each month. This study established that eye drops are the single most wasted medication, with approximately 65.7%

by volume discarded across all settings as a part of standard practice. In the outpatient setting, 56.8% of eyedrops by volume which accounts for 711 kg CO2-equivalents per month, a finding which makes our study pertinent to the overall problem of waste within ophthalmology.[19] The use of multi-use eye drops, rather than single use, has alone shown to be beneficial in reducing waste, though there remains room for improvement given the ongoing waste cited above.[14,19]

The goal of this study is to provide a reliable method for determining the microbial contamination rates for multi-use eye drops and to contribute to evidence in support of an approach to medication management that balances the need to protect patients from infection and efforts to reduce waste affecting the health care system and the planet. We hypothesized that there would be differences in microbial contamination by location sampled and by type of preservative present in the medication.

Methods *(≥250 words)*

Sampling method:

Eye drop medications, proparacaine, tropicamide, and fluorescein, were collected from Casey Eye Institute after 28 days of use in the outpatient setting. These medications were selected for two reasons, they are incredibly common in the outpatient setting and they contain common preservatives, benzalkonium chloride and chlorobutanol. The bottles were stored at 4° C for up to one week prior to sampling. Each bottle was sampled in the 3 ways. 2 drops (~100 µL) of eye drop solution plated neat on chocolate agar, Luria broth agar (LBA), and Sabouraud Agar (SDA) plates. Eye drop fluid was exposed to a preservative inactivator and then plated on chocolate agar, LBA, and SDA plates. For benzalkonium chloride preserved drops (proparacaine and tropicamide), eye drops were diluted 1:10 with Tryptone-Azolectin-Tween (TAT) broth containing 4% Tween 20 and 0.5% Lecithin, an inactivator of benzalkonium chloride per the US pharmacopeia recommendations.[12,20] For chlorobutanol preserved drops (fluorescein), eye drops were diluted 1:5 with PBS as dilution inactivated chlorobutanol per the US pharmacopeia recommendationsl.[20] Finally, dropper tips were removed in a sterile fashion and placed in tryptic soy broth (TSB) at 37° C on a shaker overnight & subsequent plating on chocolate agar, LBA, and SDA plates. A representative sample was taken from each of the plates that grew microbes. These colonies were cultured in TSB on a n overnight and were frozen as glycerol stocks for future identification. 16s rRNA identification was not completed with eye drop sample microbes due to time constraints but was completed as with positive controls to ensure the validity of the method.

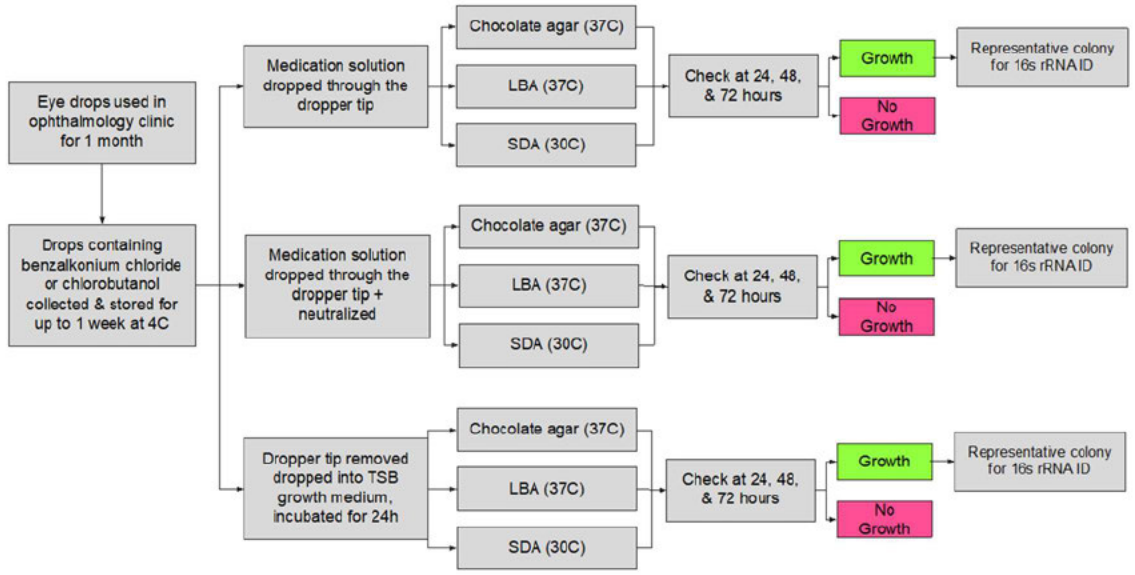

Figure 1. Experimental methods overview.

Positive control methods:

Four representative organisms, *s. aureus, p. aeruginosa, e. coli, and c. albicans,* were selected as positive controls. Organisms were acquired from the OHSU core lab. Growth curves were established to correlate optical density at 600 nanometers (OD600) with the number of colony forming units for each organism. A spectrophotometer was used to determine the OD600 of liquid culture prior to exposure to eye drop solution. Each of these organisms was exposed to the 2 preservative types, BAK and chlorobutanol in the following fashion. Each microbial species was streaked from frozen stocks and allowed to incubate for 24 hours. A single colony from each plate was selected and S aureus, P. aeruginosa, E. coli, and C. albicans were cultured in Luria broth (LB) overnight on a shaker. Prior to inoculation of eye drops, spectrophotometric measurements were taken of each of the species cultured in liquid media to estimate the CFU/mL in the inoculum. Cultures were then serially diluted 1:10 six times in PBS. Each microbial culture dilution was then combined with a newly opened sterile eye drop solution. Within 1 minute of combining the microbial culture with the eye drop, preservative neutralization was added, and the sample was immediately plated on LBA for bacteria and SDA for yeast. For benzalkonium chloride preserved drops, neutralization consisted of diluting the microbial culture + eye drop 1:10 in TAT broth.[21] For chlorobutanol preserved drops, neutralization consisted of diluting the microbial culture + eye drop 1:5 in PBS, as this degree of dilution appeared to provide reasonable conservation of bacterial growth in prior dilution experiments.[16] The microbial culture, preservative, and neutralization agent solution was then plated on LBA plates for bacteria and SDA plates for yeast. The plates were incubated for 24 hours, and colonies were counted and compared to the expected CFU based on the initial inoculum.

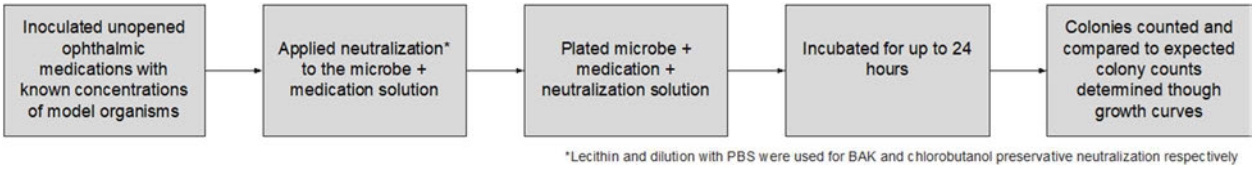

Figure 2. Positive control experiment overview.

Identification of microorganism:

S. aureus and *e. coli* were served as representative organisms to confirm the efficacy of our 16s ribosomal RNA identification protocol. Bacteria were cultured in liquid media from frozen glycerol stocks and their DNA was extracted using a TRIzol-chloroform protocol. DNA was purified using the Qiagen DNA purification kit. PCR was performed in a 50-µl total volume using variable region primers for V1-V3 and V3-V4 (Table 1) 16s rRNA genome.[22] Each reaction mixture contained 24 ng of gDNA, 1× Phusion HF buffer, 0.2 mM deoxynucleotide triphosphates (dNTPs), 0.125 µM each forward and reverse primer, 7.5% dimethyl sulfoxide (DMSO), and 0.25 µL of Phusion HF II DNA polymerase (Thermo Fisher). PCR was conditions were as follows: 98°C for 40 s, 30 cycles of 98°C for 20 s, the V-region specific annealing temperature (Table 1) for 40 s, and 72°C for 40 s, and a final extension step at 72°C for 2 min. PCR product was purified using a Qiagen PCR purification kit and sent for sanger sequencing with GENEWIZ. Resultant sequences were then queried in NCBI nucleotide BLAST.

V-region	Forward primer	Reverse primer	Forward sequence (5'–3')	Reverse sequence (5'–3')	Annealing temp (°C)
V1-V3	27F	534R	AGA GTT TGA TYM TGG CTC AG	ATT ACC GCG GCT GCT GG	57
V3-V4	341F	785R	CCT ACG GGN GGC WGC AG	GAC TAC HVG GGT ATC TAA TCC	55

Table 1. Primer sequences and annealing temperatures.[22]

Negative control methods:

Eye drops were neutralized and plated on chocolate agar, LBA, and SDA plate alongside positive control experiments. Additionally, new dropper tips were cultured in TSB overnight and the culture was subsequently plated. Neither drops nor cultured dropper tips from new bottles yielded microbial growth of any kind.

Statistical analysis:

To assess statistical significance in rates of contamination between location sampled and preservative type, a Fisher's exact test was carried out at the 5% significance level.

Results *(≥500 words)*

Positive control optimization:

Four representative, positive control organisms, *s. aureus, p. aeruginosa, e. coli, and c. albicans,* were exposed to eye drop medications containing the two preservatives of interest in this study, benzalkonium chloride (BAK) and chlorobutanol (Figure 3). The lower limit of detection was established as fewer than 10 colonies and the upper limit was established at greater than 300 colonies. *S. aureus* and *c. albicans* were successfully recovered after exposure to both BAK and chlorobutanol, though the number of colony forming units was reduced compared to what would be expected based on the initial inoculum. We were able to recover *p. aeruginosa* after exposure to BAK, though the number of CFUs was similarly reduced. We were unable to recover *p. aeruginosa* after exposure to chlorobutanol and *e. coli* after exposure to both BAK and chlorobutanol.

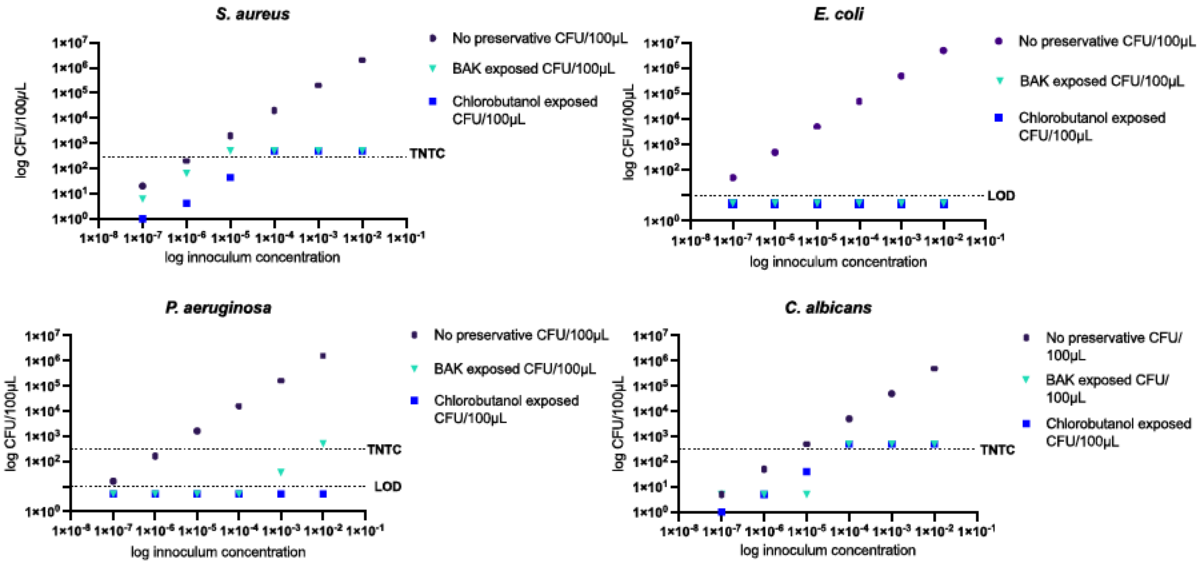

Figure 3. Positive control organisms after preservative exposure log plot. Each organism was exposed to no preservative, benzalkonium chloride (BAK), and chlorobutanol. Limit of detection (LOD) <10 colonies, too numerous to count (TNTC) >300 colonies.

Two representative, positive control organisms, *s. aureus* and *e. coli,* were used to validate 16s rRNA sequence identification. PCR product of V1-V3 variable region of the 16s rRNA genome demonstrated bands of the expected size on gel electrophoresis (Figure 4a) and nucleotide BLAST search of the sanger sequencing results accurate identification for both model organisms (Figure 4b and 4c).

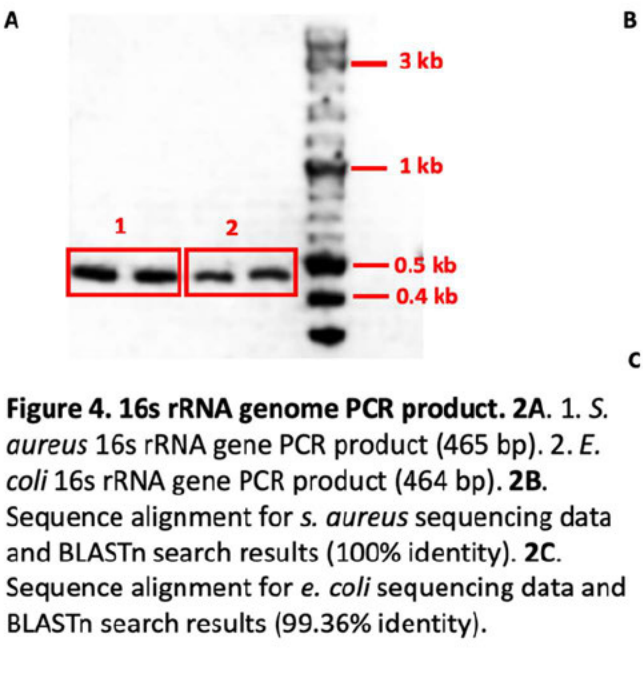

Figure 4. 16s rRNA genome PCR product. 2A. 1. *S. aureus* 16s rRNA gene PCR product (465 bp). 2. *E. coli* 16s rRNA gene PCR product (464 bp). **2B.** Sequence alignment for *s. aureus* sequencing data and BLASTn search results (100% identity). **2C**. Sequence alignment for *e. coli* sequencing data and BLASTn search results (99.36% identity).

Experimental results:

Overall, 18 multi-use eye drops were collected from a single outpatient glaucoma clinic after 28 days of use. None of the bottles had known contact with patients or providers. Fluid from all 18 eye drops was sampled according to protocol, however, 6 of the dropper tips were excluded from the data set as they were incubated in culture media that was found to have polymicrobial contamination related to the media itself. By preservative type, 13 bottles contained benzalkonium (BAK) 0.01% and 5 contained chlorobutanol 1.1%. Of note, the drops preserved with BAK were plastic with an everted dropper style and those preserved with chlorobutanol were glass with a glass eyedropper situated within the bottle and suspended in the medication solution. By medication type, there were 11 bottles containing proparacaine, 2 bottles containing tropicamide, and 5 bottles containing fluorescein.

Overall, 3 eye drops (18%) demonstrated the presence of contamination (Table 2) and each only grew a single colony. The fluid itself (drops and drops with preservative neutralization) had an 11% contamination rate and the cultured dropper tips had a 9% contamination rate (χ^2 test, p >0.9918). 18% of BAK preserved and 20% of chlorobutanol preserved drops demonstrated presence of microbial contaminants (Table 3).

#	*Medication, preservative*	*Neat*	*Drop + Neutralization*	*Cultured dropper tip*
1	Proparacaine, BAK	0	0	excluded
2	Proparacaine, BAK	0	0	excluded
3	Proparacaine, BAK	0	0	excluded
4	Proparacaine, BAK	0	0	excluded
5	Tropicamide, BAK	0	0	excluded
6	Tropicamide, BAK	0	0	excluded
7	Proparacaine, BAK	0	0	0

8	Proparacaine, BAK	0	0	0
9	Fluorescein, chlorobutanol	0	0	0
10	Fluorescein, chlorobutanol	0	0	0
11	Fluorescein, chlorobutanol	0	0	1, bacterial colony on LBA
12	Proparacaine, BAK	0	0	0
13	Proparacaine, BAK	0	0	0
14	Proparacaine, BAK	0	0	0
15	Proparacaine, BAK	1, mold on SDA	0	0
16	Proparacaine, BAK	0	1, mold on SDA	0
17	Fluorescein, chlorobutanol	0	0	0
18	Fluorescein, chlorobutanol	0	0	0

Table 2. Summary of colony counts by location sampled. Morphology and growth media noted for samples that demonstrated microbial growth.

	Growth	No Growth
Location sampled		
Drops	2 (11%)	16 (89%)
Dropper tip	1 (9%)	11 (91%)
Preservative type		
BAK	2 (18%)	11 (82%)
Chlorobutanol	1 (20%)	4 (80%)

Table 3. Summary of contamination rates. 11% of dropper fluid sample vs 9% of cultured dropper tips were contaminated (Fisher's exact test, p >0.9918). 18% of BAK preserved vs 20% of chlorobutanol preserved medications were contaminated (Fisher's exact test, p = 0.4129).

Discussion *(≥500 words)*

By providing a method for assessing contamination rates for two locations on multi use eye drops, the medication fluid and the dropper tip, we accounted for 2 of the possible methods of transmission of microbes from the bottle to the patient, via medication solution passing through the dropper tip and via dropper tip contact with the patient. While some studies opted to sample residual medication solution passing it through the tip [3,23], many chose to aspirate the solution in a sterile in a sterile fashion. [5,21,24] We posit that some of the disagreement in contamination rates may arise from the fact that different sampling methods yield different results. However, we feel that our study provides a clinically relevant method.

Finally, the positive control experiments in this study demonstrated that both benzalkonium chloride and chlorobutanol have robust efficacy against common microorganisms, as there was a universal reduction in the number of organisms recovered even after very short exposure to these preservatives. In general, this supports the efficacy of these preservatives and safety of multi-use eye drops.[23,25] However, our findings must be taken in context as they may represent strains that are less resistant to preservatives in multi-use eye drops. The literature suggests that organisms have developed resistance to these preservatives, particularly benzalkonium chloride, an important factor not accounted for in our positive control model. [23,26,27]

The average contamination of rate in this study (18%) falls around the average of other studies conducted in the outpatient clinical setting[3,5,24], though this is difficult to interpret in the setting of a small sample size and the inclusion of only 3 medication and 3 preservative types. The microbial burden was very low as each contaminated drop only grew a single colony forming unit (CFU). The literature varies when it comes to the number and variety of microbes found on each individual bottle. Several studies found that most eye drop bottle yielded only one pathogen regardless of site sampled, though

there have been instances where numerous CFU were recovered and polymicrobial contaminants observed, though this appears to be more common in multi-use medications for home use than in those used in outpatient or inpatient settings.[3,5,23-25]

Several studies note that dropper design may impact the likelihood of contamination, though there is lack of agreement about what types of containers result in higher rates of contamination and which part of the eye drop bottle is most likely to harbor microbial contaminants, though many cite the dropper tip as the most commonly contaminated [3,11-13] In our study, dropper tips were more likely to have microbial contamination than the medication fluid itself, though this result was not statistically significant. Interestingly, there was no concordance between presence of microbes on cultured dropper tips and in dropper fluid. These results are similar to those reported in earlier studies and may support the previously described self-sterilizing effect of many eye drops.[11,28]

Finally, our study found that there was no statistical difference between contamination rates of eye drops preserved with BAK and chlorobutanol, though it is difficult to draw conclusions due to small sample size. Few other studies have compared contamination rates based on preservatives as an independent variable, and have primarily focused on differences between medications.[5,29,30] However, those that have focused on difference in contamination between preservatives have suggested that chlorobutanol preserved multi-use solutions are more likely to be contaminated than BAK preserved solutions.[24]

This study demonstrated a method for isolating and identifying contaminants from expired multi-use eye drops, information we hope will be used to further investigate the true incidence of microbial contamination of multi-use eye drops in the outpatient setting in order to develop disposal guidelines that strike a balance between patient safety and waste reduction efforts, a worthy endeavor considering the impact of medical waste on our planet and the downstream morbidity and mortality of climate change.[17,19]

Conclusions *(2-3 summary sentences)*

This study demonstrates a method by which contaminants can be isolated and identified from multi-use eye drops. The preliminary results suggest that the presence of microbes on the dropper tip does not necessarily lead to contamination of the medication itself, even if the medication solution is passed through the contaminated dropper tip and that the burden of microbial contaminants is low. Limitations include small sample size and small number medications and preservative types sampled.

References *(JAMA style format)*

1. Tsegaw A, Abula T, Assefa Y. Bacterial Contamination of Multi-dose Eye Drops at Ophthalmology Department, University of Gondar, Northwest Ethiopia. *Middle East Afr J Ophthalmol*. 2017 Apr-Jun 2017;24(2):81-86. doi:10.4103/meajo.MEAJO_308_16
2. Jokl DH, Wormser GP, Nichols NS, Montecalvo MA, Karmen CL. Bacterial contamination of ophthalmic solutions used in an extended care facility. *Br J Ophthalmol*. Oct 2007;91(10):1308-10. doi:10.1136/bjo.2007.115618
3. Nentwich MM, Kollmann KH, Meshack J, Ilako DR, Schaller UC. Microbial contamination of multi-use ophthalmic solutions in Kenya. *Br J Ophthalmol*. Oct 2007;91(10):1265-8. doi:10.1136/bjo.2007.116897
4. Schein OD, Hibberd PL, Starck T, Baker AS, Kenyon KR. Microbial contamination of in-use ocular medications. *Arch Ophthalmol*. Jan 1992;110(1):82-5. doi:10.1001/archopht.1992.01080130084030
5. Teuchner B, Wagner J, Bechrakis NE, Orth-Höller D, Nagl M. Microbial contamination of glaucoma eyedrops used by patients compared with ocular medications used in the hospital. *Medicine (Baltimore)*. Feb 2015;94(8):e583. doi:10.1097/MD.0000000000000583
6. Schein OD, Wasson PJ, Boruchoff SA, Kenyon KR. Microbial keratitis associated with contaminated ocular medications. *Am J Ophthalmol*. Apr 15 1988;105(4):361-5. doi:10.1016/0002-9394(88)90298-x
7. Mayo MS, Schlitzer RL, Ward MA, Wilson LA, Ahearn DG. Association of Pseudomonas and Serratia corneal ulcers with use of contaminated solutions. *J Clin Microbiol*. Aug 1987;25(8):1398-400. doi:10.1128/jcm.25.8.1398-1400.1987
8. Porges Y, Rothkoff L, Glick J, Cohen S. Sterility of glaucoma medications among chronic users in the community. *J Ocul Pharmacol Ther*. Apr 2004;20(2):123-8. doi:10.1089/108076804773710795
9. Penland RL, Wilhelmus KR. Stenotrophomonas maltophilia ocular infections. *Arch Ophthalmol*. Apr 1996;114(4):433-6. doi:10.1001/archopht.1996.01100130429013
10. Brudieu E, Duc DL, Masella JJ, et al. [Bacterial contamination of multi-dose ocular solutions. A prospective study at the Grenoble Teaching Hospital]. *Pathol Biol (Paris)*. Dec 1999;47(10):1065-70.
11. Geyer O, Bottone EJ, Podos SM, Schumer RA, Asbell PA. Microbial contamination of medications used to treat glaucoma. *Br J Ophthalmol*. Apr 1995;79(4):376-9. doi:10.1136/bjo.79.4.376
12. Fazeli MR, Nejad HB, Mehrgan H, Elahian L. Microbial contamination of preserved ophthalmic drops in outpatient departments: Possibility of

an extended period of use. *DARU journal of pharmaceutical sciences*. 2004;12(4):151-155.
13. Raynaud C, Laveran H, Rigal D, Bonicel P. [Bacterial contamination of eyedrops in clinical use]. *J Fr Ophtalmol*. 1997;20(1):17-24.
14. Jensen MK, Nahoopii R, Johnson B. Using multidose eyedrops in a health care setting: a policy and procedural approach to safe and effective treatment of patients. *JAMA Ophthalmol*. Dec 2014;132(12):1476-9. doi:10.1001/jamaophthalmol.2014.3248
15. Infection Prevention in Eye Care Services and Operating Areas and Operating Rooms - 2012. . American Academy of Ophthalmology. https://www.aao.org/clinical-statement/infection-prevention-in-eye-care-services-operatin.
16. Pharmacopeia U. USP Reference Standards. Updated January 4, 2013. Accessed October 23, 2020. http://www.usp.org/reference-standards
17. Chung JW, Meltzer DO. Estimate of the carbon footprint of the US health care sector. *JAMA*. Nov 2009;302(18):1970-2. doi:10.1001/jama.2009.1610
18. Centers for Medicare & Medicaid Services National health expenditure data. Updated Modified March 24, 2020. Accessed October 23, 2020. https://www.cms.gov/Research-Statistics-Data-and-Systems/Statistics-Trends-and-Reports/NationalHealthExpendData/NHE-Fact-Sheet.html .
19. Tauber J, Chinwuba I, Kleyn D, Rothschild M, Kahn J, Thiel CL. Quantification of the Cost and Potential Environmental Effects of Unused Pharmaceutical Products in Cataract Surgery. *JAMA*

Ophthalmol. Oct 2019;137(10):1156-1163. doi:10.1001/jamaophthalmol.2019.2901
20. Pharmacopeia U. Validation of microbial recovery from pharmacopeial articles <1227>. In: US Pharmacopeia 25/National Formulary 20. Washington, DC: United States Pharmacopeial Convention20011. p. 2259-2261.
21. Livingstone DJ, Hanlon GW, Dyke S. Evaluation of an extended period of use for preserved eye drops in hospital practice. *Br J Ophthalmol*. May 1998;82(5):473-5. doi:10.1136/bjo.82.5.473
22. Abellan-Schneyder I, Matchado MS, Reitmeier S, et al. Primer, Pipelines, Parameters: Issues in 16S rRNA Gene Sequencing. *mSphere*. Feb 24 2021;6(1)doi:10.1128/mSphere.01202-20
23. Tamrat L, Gelaw Y, Beyene G, Gize A. Microbial Contamination and Antimicrobial Resistance in Use of Ophthalmic Solutions at the Department of Ophthalmology, Jimma University Specialized Hospital, Southwest Ethiopia. *Can J Infect Dis Med Microbiol*. 2019;2019:5372530. doi:10.1155/2019/5372530
24. Bachewar P, Deshmukh N, Choudhari D, Sachin R., Joshi RS. Evaluation of used eye drop containers for microbial contamination in outpatient department of tertiary care teaching hospital. *International Journal of Basic & Clinical Pharmacology*. 2018-04-23 2018;7(5):895. doi:10.18203/2319-2003.ijbcp20181631
25. Rahman MQ, Tejwani D, Wilson JA, Butcher I, Ramaesh K. Microbial contamination of preservative free eye drops in multiple application containers. *Br J Ophthalmol*. Feb 2006;90(2):139-41. doi:10.1136/bjo.2005.078386
26. Kim M, Weigand MR, Oh S, et al. Widely Used Benzalkonium Chloride Disinfectants Can Promote Antibiotic Resistance. *Appl Environ Microbiol*. Sep 01 2018;84(17)doi:10.1128/AEM.01201-18
27. Nordholt N, Kanaris O, Schmidt SBI, Schreiber F. Persistence against benzalkonium chloride promotes rapid evolution of tolerance during periodic disinfection. *Nat Commun*. Nov 23 2021;12(1):6792. doi:10.1038/s41467-021-27019-8
28. Høvding G, Sjursen H. Bacterial contamination of drops and dropper tips of in-use multidose eye drop bottles. *Acta Ophthalmol (Copenh)*. Apr 1982;60(2):213-22. doi:10.1111/j.1755-3768.1982.tb08375.x
29. Clark PJ, Ong B, Stanley CB. Contamination of diagnostic ophthalmic solutions in primary eye care settings. *Mil Med*. Jul 1997;162(7):501-6.
30. Wessels IF, Bekendam P, Calvin WS, Zimmerman GJ. Open drops in ophthalmology offices: expiration and contamination. *Ophthalmic Surg Lasers*. 1999 Jul-Aug 1999;30(7):540-6.