Component Analysis of Multipurpose Contact Lens Solutions To Enhance Activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus*

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More than 125 million people wear contact lenses worldwide, and contact lens use is the single greatest risk factor for developing microbial keratitis. We tested the antibacterial activity of multipurpose contact lens solutions and their individual component preservatives against the two most common pathogens causing bacterial keratitis, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The *in vitro* antibacterial activity of five multipurpose contact lens solutions (Opti-Free GP, Boston Simplus, Boston Advance, Menicare GP, and Lobob) was assayed by the standard broth dilution method. Synergy between the preservative components found in the top performing solutions was assayed using checkerboard and time-kill assays. The ISO 14729 criteria and the standard broth dilution method were used to define an optimized contact lens solution formulation against a clinical panel of drug-susceptible and drug-resistant *P. aeruginosa* and *S. aureus* strains. Preservatives with the biguanide function group, chlorhexidine and polyaminopropylbiguanide (PAPB), had the best antistaphylococcal activity, while EDTA was the best antipseudomonal preservative. The combination of chlorhexidine and EDTA had excellent synergy against *P. aeruginosa*. A solution formulation containing chlorhexidine (30 ppm), PAPB (5 ppm), and EDTA (5,000 ppm) had three to seven times more antipseudomonal activity than anything available to consumers today. A multipurpose contact lens solution containing a combination of chlorhexidine, PAPB, and EDTA could help to reduce the incidence of microbial keratitis for contact lens users worldwide.

**MATERIALS AND METHODS**

**Bacterial strains.** *P. aeruginosa* strains PAO1 and PA103 were obtained from the American Type Culture Collection (ATCC) and multidrug-resistant (MDR) *P. aeruginosa* strain P4 from a tertiary care academic hospital in New York. *S. aureus* strains were methicillin-resistant *S. aureus* (MRSA) TCH 1516 (USA300) from ATCC, Sanger 252 (USA200) from the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARS), and methicillin-susceptible *S. aureus* UAMS1 from G. Somerville (University of Nebraska). The following fluoroquinolone-susceptible (FQs) or fluoroquinolone-resistant (FQR) clinical keratitis isolates were obtained from the collection of the Charles T. Campbell Ophthalmic Microbiology Laboratory at the University of Pittsburgh: *S. aureus* K2751 (FQs), K2738 (FQR), and K2735 (FQR) and *P. aeruginosa* K2749 (FQs), PA13 (FQR), and PA16 (FQR).

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Preservatives and reagents. Mueller-Hinton broth (MHB; Spectrum Chemicals) was supplemented with CaCl₂ and MgCl₂ to make cation-adjusted MHB (Ca-MHB) (final cation concentrations, 20 to 25 mg/liter Ca²⁺ and 10 to 12.5 mg/liter Mg²⁺). Other reagents were obtained from the following vendors: Luria Broth base (LB) from Hardy Diagnostics; Todd-Hewitt Broth base (THB) from Neogen; EDTA from Sigma; chlorhexidine gluconate (CHD) from Sigma; polyaminopropyl biguanide (PAPB) from Lotioncrafter; resazurin sodium from Sigma; and Difco D/E neutralization broth from BD.

Multipurpose contact lens solutions. Opti-Free GP (Alcon), Boston Simplus and Boston Advance (Bausch & Lomb), Menicare GP (Menicon), and Lobob (Lobob Labs) were purchased from Amazon. These contact lenses were chosen because they represent a leading extended-wear silicon hydrogel lens approved by the FDA for up to 14 days of extended wear.

Determination of MICs. MIC values for contact lens solutions and their preservative components were determined using broth microdilution in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines (8). Bacterial viability was determined using an optical plate reader (at an optical density at 600 nm [OD₆₀₀]) and resazurin indicator dye as previously described (9).

For individual contact lens solution preservatives such as CHD, PAPB, EDTA, and benzyl alcohol, concentrated stocks were purchased and then diluted in sterile water to 10 times the concentrations used in the contact lens solutions. The broth microdilution method outlined in the CLSI guidelines mentioned above was applied as follows: in the first row of wells in a 96-well plate, we added 20 μl of 5 × 10⁴ CFU/ml of bacteria suspended in Ca-MHB, 20 μl of the 10× preservative, and 160 μl of Ca-MHB. Thus, the first row of wells on the 96-well plate contained 200 μl of liquid in total with 5 × 10⁴ CFU/ml bacteria and a 1× concentration of the preservative. Serial dilutions were performed by transferring 100 μl from the first well into 100 μl of Ca-MHB with 5 × 10⁵ CFU/ml bacteria in the second row of wells, mixing well, and then repeating down the rows. MIC testing of the contact lens solutions and our optimized formulation was performed using an adaptation of the CLSI broth microdilution guidelines for antibiotics. In the first row of wells in the 96-well plate, 20 μl of 5 × 10⁴ CFU/ml of bacteria suspended in Ca-MHB was combined with 180 μl of contact lens solution. Thus, the first well contained 10% Ca-MHB and 90% contact lens solution with 5 × 10⁵ CFU/ml bacteria. Serial dilutions were performed by transferring 100 μl from the first well into 100 μl of Ca-MHB with 5 × 10⁵ CFU/ml bacteria in the second row of wells, mixing well, and then repeating down the rows. Thus, going down a vertical column of wells in the 96-well plate, every well would have 5 × 10⁵ CFU/ml bacteria, followed by a decreasing percentage (90%, 45%, 22.5%, 11.3%, 5.6%, 2.8%, 1.4%, or 0.7%) of contact lens solution and a corresponding increase in the percentage of Ca-MHB.

Traditional checkerboard and time-kill assays. Traditional checkerboard and time-kill assays were performed as previously described (10). Overnight cultures of P. aeruginosa (in LB) and S. aureus (in THB) were grown at 37°C, pelleted, washed twice, and resuspended in phosphate-buffered saline (PBS) to an OD₆₀₀ of 0.40. Bacterial stocks were then diluted in Ca-MHB to an initial inoculum of ~1 × 10⁸ CFU/ml and contact lens solution preservatives added at the indicated concentrations. For the checkerboard assay, 96-well plates were incubated with shaking at 37°C for 20 h, the OD₆₀₀ was monitored, resazurin was added (final concentration, 3.38 ng/ml), and color changes were assessed after 24 h of incubation at 37°C. For the time-kill assay, 96-well plates were incubated with shaking at 37°C. Aliquots (20 μl) of test solutions were taken at the indicated time points, serially diluted, and plated for CFU enumeration.

ISO 14729 assay. The ISO 14729 assay was performed as previously described (11). Briefly, 500 μl of washed and concentrated bacteria was added to 4,500 μl PBS containing CHD (30 ppm), PAPB (5 ppm), and EDTA (5,000 ppm) to reach a concentration of 1 × 10⁴ CFU/ml, mixed, and incubated for 1 h at room temperature. A 100-μl volume of test solution was removed, serially diluted in Dey-Engley neutralizing broth, and plated for CFU enumeration.

Evaluation of the effect of the C30/P5/E5000 formulation on contact lens biofilms. Biofilm studies employed a published technique (12). Briefly, senofilcon A lenses were washed with PBS and then placed in 12-well tissue culture plates with 4 ml of bacterial cell suspensions; overnight cultures were washed twice with PBS and diluted in PBS to reach an absorbance value of 0.1 at 660 nm. Lenses were incubated at 37°C for 120 min to allow adhesion of bacteria to the lens surface (adherence phase). Lenses were then transferred to new 12-well plates containing 4 ml of fresh PBS. Each lens was then placed in an Eppendorf tube filled with 2 ml of 1% THB or 1% (wt/vol) LB for S. aureus or P. aeruginosa, respectively, and was rotated at 37°C for 24 h (biofilm formation phase). Each lens was then washed in fresh PBS for 5 s to simulate the rinsing step and placed in 4 ml of CHD at 30 ppm, PAPB at 5 ppm, and EDTA at 5,000 ppm (C30/P5/ E5000) or 4 ml of a PBS control and incubated at room temperature for 4 h. Lenses were washed again in fresh PBS for 5 s and transferred to a 1.5 ml Eppendorf tube containing 1 ml of PBS and 1-mm-diameter silicon beads. In order to break up the biofilm on the contact lenses, the tubes were rigorously shaken at 6,000 rpm for 1 min twice, with 1 min of cooling down on ice between agitations. The bacterial suspensions were serially diluted in Dey-Engley neutralizing broth, and serial dilutions were plated on THB and LB agar plates for S. aureus and P. aeruginosa, respectively, to evaluate viability.

RESULTS
Activity of five commercial contact lens solutions against MRSA and P. aeruginosa. We tested five multipurpose contact lens solutions from major manufacturers in the United States: Boston Simplus, Boston Advance, Opti-Free, Menicare GP, and Lobob. The antibacterial preservatives found in each solution are listed in Fig. 1A. The MIC of each solution against methicillin-resistant S. aureus (MRSA) TCH 1516 and P. aeruginosa PA01 was determined by CLSI broth microdilution methodology (8). Boston Simplus had the most potent antistaphylococcal activity, with a MIC of 1.5% (Fig. 1B), while Menicare GP had the most potent antipseudomonal activity, with a MIC of 23% (Fig. 1C). All multipurpose solutions tested were less effective against P. aeruginosa than against MRSA. The same results were observed when we tested the multipurpose contact lens solutions against three S. aureus and three P. aeruginosa clinical keratitis isolates (see Fig. S1 in the supplemental material).

Preservatives with a biguanide functional group have the highest anti-MRSA activity, while EDTA has the highest antipseudomonal activity. We sought to determine which preservative(s) found in each top performing solution yielded the antibacterial effects observed. Boston Simplus, with the highest anti-MRSA activity, utilizes the biguanide-containing preservatives CHD and PAPB. CHD and PAPB were equally active against MRSA, with MICs of 2.5 ppm and 2.5 ppm, and were less active against P. aeruginosa, with MICs of 15 ppm and 20 ppm, respectively. No synergy of CHD and PAPB in combination was observed for either MRSA or P. aeruginosa (Fig. 1D). Menicare GP, the most active solution against P. aeruginosa, utilizes EDTA and benzyl alcohol as preservatives. The MICs of EDTA were 2,500 ppm against P. aeruginosa and 300 ppm against MRSA. The MICs of benzyl alcohol were 5,000 ppm against P. aeruginosa and 10,000 ppm against MRSA. Synergy of EDTA and benzyl alcohol was observed against P. aeruginosa but not against MRSA (Fig. 1E).

CHD and EDTA are synergistic against P. aeruginosa. Using checkerboard assays to test combinations of component pre-
servative found in Boston Simplus and Menicare GP, we dis-
covered that the most potent synergistic combination against
*P. aeruginosa* was CHD plus EDTA (Fig. 2A). Used together, a
solution of 4 ppm CHD (1/10 MIC) plus 300 ppm EDTA
(1/8 MIC) was sufficient to eradicate *P. aeruginosa*, with a
corresponding fractional inhibitory concentration index of
0.39. The bactericidal activity of this combination was ex-
tremely rapid, with a 4 log₁₀ reduction in *P. aeruginosa*
numbers in 2 h in quantitative killing assays (Fig. 2B). Synergy of
CHD plus EDTA was also observed against three *P. aeruginosa*
clinical keratitis isolates, with fraction inhibitory concentra-
tion index values of less than 0.13 as calculated by checker-
board assays (see Fig. S2 in the supplemental material).

A formulation of CHD, PAPB, and EDTA showed excellent
antibacterial activity against MRSA and *P. aeruginosa*. The
combination of CHD and PAPB in Boston Simplus had strong
activity against MRSA (Fig. 1B), but the EDTA concentration in
this product is too low for synergy against *P. aeruginosa*. Such
synergy was achieved by combining the EDTA concentration of
Menicare GP with the CHD and PAPB concentrations of Boston
Simplus. A formulation of CHD at 30 ppm, PAPB at 5 ppm, and
EDTA at 5,000 ppm (C30/P5/E5000) satisfies the international
criteria for contact lens solution efficacy against bacterial patho-
gens described in ISO 14729. In just 1 h, the concentrations of
MRSA and *P. aeruginosa* were reduced by >4 log₁₀ (Fig. 2C),
which was far less than the manufacturer’s recommended disin-
fec tion time for either Boston Simplus (4 h) or Menicare GP (6 h).
A >4 log₁₀ reduction in CFU per milliliter was also observed after
just 1 h against all six clinical keratitis isolates (see Fig. S3 in the
supplemental material). The C30/P5/E5000 formulation was also
effectively extending against a panel of clinical *S. aureus* and *P.
aeruginosa* isolates, including MRSA and multidrug-resistant *P.
aeruginosa*, as well as against our six clinical keratitis strains. The
favorable MIC of C30/P5/E5000 was 3% to 6% against all strains
tested (Fig. 2D).

The C30/P5/E5000 formulation is able to eradicate *S. aureus*
and *P. aeruginosa* biofilms that have formed on contact lens
surfaces. Among all isolates or our clinical keratitis isolates, *S.
aureus* K2738 and *P. aeruginosa* K2749 were the most mucoid and
robust biofilm producers. We used a previously published proto-
col (12) to grow mature *S. aureus* K2738 and *P. aeruginosa* K2749
biofilms on a popular brand of silicon hydrogel lenses. Treatment
of these biofilm-coated contact lenses with C30/P5/E5000 for 4 h
at room temperature, the minimum recommended disinfection
time for most multipurpose contact lens solutions, resulted in
a >4 log₁₀ reduction in the numbers of viable *S. aureus* and *P.
aeruginosa* (Fig. 3).
DISCUSSION

With millions of daily users, contact lens-related microbial keratitis continues to be a significant health problem. Contact lenses interfere with several innate immune defense mechanisms of the eye (13). Furthermore, most contact lens users are noncompliant with proper lens cleaning and care procedures (14), with significant percentages reporting reuse of old contact lens solution or topping off their existing solution each night. In this setting, a contact lens solution with rapid killing activity against the major keratitis-causing pathogens, even when diluted significantly, could reduce the incidence of keratitis. By harnessing the synergy of CHD and EDTA against *P. aeruginosa* (15, 16) and EDTA is effective against *P. aeruginosa* biofilms and *S. aureus* biofilms (17–19), a C30/P5/E5000 formulation could provide a one-step solution to reducing contact lens-related keratitis of all causes.

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