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Killing efficacy of a new hypothermic corneal storage medium against the micro-organisms frequently found in human donor cornea intended for transplantation

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ABSTRACT

Objective To study the in vitro killing efficacy of Kerasave (AL.CHI.MI.A Srl), a medium provided with amphotericin B tablet for hypothermic storage of human donor corneas, against relevant contaminants associated with postkeratoplasty infections.

Methods and Analysis The antimicrobial activity of Kerasave was determined after 0, 3 and 14 days of incubation at 2°C–8°C, inoculating Kerasave and the control medium with 10^5–10^6 colony forming units (CFU) of *Candida albicans* (CA), *Fusarium solani* (FS), *Aspergillus brasiliensis* (AB), *Staphylococcus aureus* (SA), *Enterococcus faecalis* (EF), *Bacillus subtilis spizizenii* (BS), *Pseudomonas aeruginosa* (PA), *Enterobacter cloacae* (EC) and *Klebsiella pneumoniae* (KP). Log_{10} reductions at different time intervals were determined by assessing the number of viable CFU using the serial dilution plating technique.

Results After 3 days, Kerasave induced the highest log_{10} decrease in the concentrations of KP, PA, CA and EC (5.37, 4.15, 2.97 and 2.67, respectively; all p<0.001). The log_{10} decreases of SA and EF were 2.27 and 2.11, respectively (all p<0.001). The lowest log_{10} decrease was observed in BS, AB and FS concentrations (0.25, 0.30 and 0.67, respectively; p<0.001 for BS and AB and p=0.004 for FS). After 14 days, the microbial count of CA, FS, SA, EF, PA and EC further decreased (p=0.006 for FS; p<0.001 for the others).

Conclusion Kerasave effectively reduced or kept unchanged the microbial concentration of almost all tested strains after 3 days. Thus, this novel medium represents a valuable tool to control the microbial contamination of human donor corneas during hypothermic storage for up to 14 days before transplantation.

Key messages

What is already known about this subject?
► Currently available hypothermic storage media are supplied with antibacterial agents to help reduce the risk of postkeratoplasty bacterial infections, but do not contain antifungal agents.

What are the new findings?
► Kerasave is a new amphotericin B-provided medium for hypothermic storage.
► As shown in in vitro time-kill tests against selected Gram-positive and Gram-negative bacteria and fungi commonly involved in postkeratoplasty infections, Kerasave effectively reduced or kept unchanged the microbial concentration after 3 days.

How might these results change the focus of research or clinical practice?
► This study confirms the killing of the most common contaminants under hypothermic corneal storage even after short incubation times and suggests this medium as a valuable tool to control the microbial contamination of human donor corneas during hypothermic storage before transplantation.

INTRODUCTION

The human donor cornea is the most commonly transplanted tissue worldwide. The use of lamellar grafts (eg, Descemet's stripping automated endothelial keratoplasty and Descemet's membrane endothelial keratoplasty) for replacing only the diseased portion of the cornea while sparing the healthy tissue has led to a significant improvement in graft survival and patient outcome.[1–3] However, corneal transplantation is still associated with a certain risk of postkeratoplasty infections.[4 5] Particularly, a trend towards an increase in the incidence of postkeratoplasty fungal infections was observed in the USA.[6 7] These cases are mainly related to the procedures of lamellar keratoplasties.[8–10]

Most European eye banks store corneal tissues in organ culture media at 31°C, containing both antibiotics and antifungal agents to help reduce the risk of microbial proliferation.[2 11] Contrarily, eye banks in North America preserve donor corneas at 2°C–8°C, and the currently available hypothermic

storage media in the US market are supplied with antibacterial agents[12] but do not contain antifungal agents.[7 13 14] For this reason, the possibility of implementing hypothermic corneal storage media with antifungals has a growing interest.[6 13–16]

Kerasave (AL.CHI.MI.A Srl, Italy) is a newly developed medical device and Conformité Européenne (CE)-marked hypothermic storage medium containing amphotericin B, which is intended for storage of human donor corneas at 2°C–8°C for up to 14 days.[15] Kerasave's Food and Drug Administration approval is pending at the time of this submission.

Previous studies provided evidence of the efficacy and safety of antifungal agents, including amphotericin B, in reducing *Candida* species contamination of Optisol-GS (Bausch & Lomb, New Jersey, USA) under hypothermic storage conditions.[13 14] More recently, Tran *et al*[17] investigated the efficacy and safety of various concentrations of amphotericin B, including 2.5 µg/mL, under hypothermic storage conditions. Our previous studies[15 18] evaluated the antimycotic efficacy of Kerasave at 2°C–8°C against *Candida albicans*, *C. tropicalis* and *C. glabrata*.

The present study aimed to assess the *in vitro* killing efficacy of Kerasave at 2°C–8°C against some clinically relevant contaminants of donor corneas, such as Gram-positive *Staphylococcus aureus*,[5 19–25] *Enterococcus faecalis*[23 24] and *Bacillus subtilis spizienii*[22]; Gram-negative *Pseudomonas aeruginosa*,[5 20 22 24] *Enterobacter cloacae*[23] and *Klebsiella pneumoniæ*[21]; and the fungi *C. albicans*,[5 21 23–25] *Fusarium solani*[5] and *Aspergillus brasiliensis*.[20]

MATERIALS AND METHODS
Experimental design
Figure 1 presents the experimental design of the time-kill assay of the present study.

Media and devices
Kerasave is a Dulbecco's Modified Eagle's Medium-based culture medium containing 6% dextran, 100 µg/mL gentamicin sulfate and 200 µg/mL streptomycin sulfate. It is supplied with an amphotericin B tablet to be added to the 20 mL medium before use (final concentration of 2.5 µg/mL). All vials of Kerasave and blisters containing amphotericin B tablets were stored at 2°C–8°C and protected from light. Each Kerasave sample was prepared under sterile conditions immediately before use by dissolving one tablet of amphotericin B in the 20 mL vial containing the Kerasave solution.

RESEP is a syringe-like, patented, CE-marked device containing a resin mixture to remove antimicrobials from the samples.[26] It has been validated for total elimination of antimicrobials from corneal storage media,[27–29] including Kerasave (online supplemental tables 1 and 2). The validation of the method for removing antimicrobial residues from Kerasave was performed according to the experimental design reported in online supplemental figure 1. In this study, 6 mL of each sample were withdrawn using the RESEP syringe and stirred at room
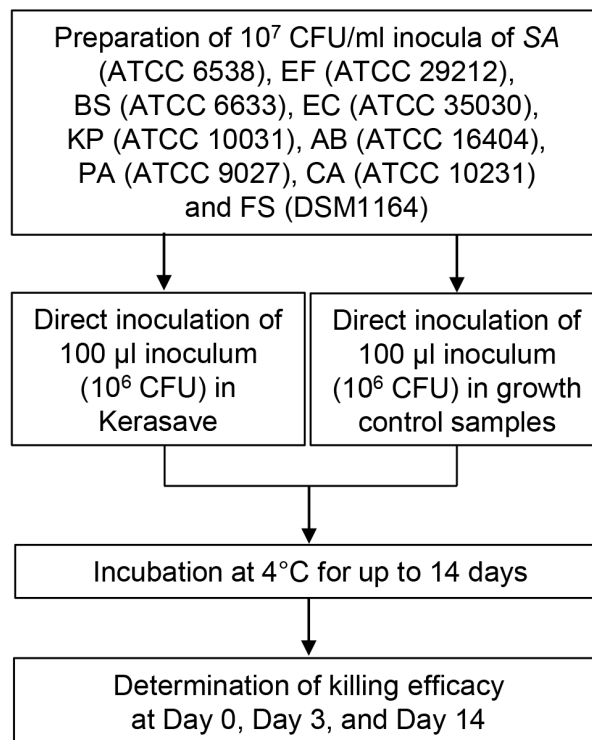
Kerasave time-kill study

Preparation of 10^7 CFU/ml inocula of *SA* (ATCC 6538), EF (ATCC 29212), BS (ATCC 6633), EC (ATCC 35030), KP (ATCC 10031), AB (ATCC 16404), PA (ATCC 9027), CA (ATCC 10231) and FS (DSM1164)

↓

Direct inoculation of 100 µl inoculum (10^6 CFU) in Kerasave | Direct inoculation of 100 µl inoculum (10^6 CFU) in growth control samples

↓

Incubation at 4°C for up to 14 days

↓

Determination of killing efficacy at Day 0, Day 3, and Day 14

Figure 1 Time-kill experimental design. In the time-kill study, 20 mL of Kerasave and growth controls were inoculated with 10^6 CFU of tested micro-organisms and incubated at 2°C–8°C for up to 14 days. The effective concentration of each micro-organism was determined after 0, 3 and 14 days of incubation. AB, *Aspergillus brasiliensis*; BS, *Bacillus subtilis spizienii*; CA, *Candida albicans*; CFU, colony forming unit; EC, *Enterobacter cloacae*; EF, *Enterococcus faecalis*; FS, *Fusarium solani*; KP, *Klebsiella pneumoniae*; PA, *Pseudomonas aeruginosa;* SA, *Staphylococcus aureus*; ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen.

temperature (RT) for 45 min. Afterwards, the samples were transferred into sterile vials.

Preparation of inocula and growth controls
S. aureus (American Type Culture Collection, ATCC 6538), *E. faecalis* (ATCC 29212), *B. subtilis spizizenii* (ATCC 6633), *E. cloacae* (ATCC 35030), *K. pneumoniae* (ATCC 10031), *A. brasiliensis* (ATCC 16404), *P. aeruginosa* (ATCC 9027) and *C. albicans* (ATCC 10231) were obtained from Microbiologics (Minnesota, USA). *F. solani* (Deutsche Sammlung von Mikroorganismen, DSM 1164) was supplied by Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Germany.

Inocula of *S. aureus, E. faecalis, B. subtilis, E. cloacae, A. brasiliensis, P. aeruginosa, C. albicans, K. pneumoniae* and *F. solani* were prepared from lyophilised pellets according to the manufacturer's protocols or from fresh culture according to McFarland standards to obtain 1.0×10^7 colony forming units (CFU/mL in physiological solution

(0.9% (w/v) NaCl); Merck-Sigma-Aldrich, Darmstadt, Germany.

Tryptone soya broth (20 mL; Bioculturalab, Italy) was used as growth control for *C. albicans*, *A. brasiliensis* and *F. solani*, and thioglycolate medium (20 mL; Bioculturalab) was used as growth control for *S. aureus*, *B. subtilis*, *P. aeruginosa* and *K. pneumoniæ*. Nutrient broth (Nutrient Broth, 20 mL; BioLife, Italy) medium was used as growth control for *E. faecalis* and *E. cloacae*.

Time-kill method

Triplicates of Kerasave samples and growth controls were inoculated with 100 µL of inoculum to obtain a final strain concentration of 10^5–10^6 CFU/mL for each time point. Effective initial strain concentration was determined by counting the number of viable CFU in the 100 µL inoculum using the serial dilution plating technique within 3 hours from spiking (day 0).

On day 3 and day 14, all Kerasave samples, except those containing *F. solani*, were treated with RESEP before plating to eliminate the antimicrobial residues. Then, the samples were serially diluted in physiological solution and plated on agar plates for viable colony counting. *C. albicans* and *A. brasiliensis* were plated on Sabouraud Chloramphenicol Agar Medium (BioLife); *S. aureus*, *B. subtilis*, *P. aeruginosa* and *K. pneumoniæ* were plated on Trypticase Soy Agar Medium (BioLife); and *E. faecalis* and *E. cloacae* were plated on nutrient agar dehydrated medium (BioLife).

On day 3 and day 14, 1 mL of Kerasave containing *F. solani* was serially diluted in physiological solution and plated on potato glucose agar dehydrated medium (Merck-Sigma-Aldrich). All growth controls were antimicrobial-free, so treatment with RESEP was skipped. At the selected time points, 1 mL of each growth control was diluted in physiological solution and plated on Petri dishes containing appropriate agar medium as described above. For each condition (i.e., after 3 days and 14 days of incubation at 2°C–8°C of Kerasave samples and growth controls, respectively), the concentration of each strain was determined by counting the number of viable CFU in 1 mL of each sample using the serial dilution plating technique.

Agar plates containing *S. aureus*, *B. subtilis*, *P. aeruginosa*, *K. pneumoniæ*, *E. faecalis* and *E. cloacae* were incubated at 30°C–35°C for 24 hours. Plates containing *C. albicans* and *A. brasiliensis* were incubated at 20°C–25°C for up to 72 hours and plates containing *F. solani* at 25°C for 5–7 days.

The number of CFU/mL in the tested sample corresponding to each plate at each time point was determined using the following formula: CFU/mL=CFU plate/dilution factor.

Statistical analysis

In previous studies,[18] the effects observed between the two experimental conditions, Kerasave versus control, were considered 'high' according to the definition by Cohen.[30] A preliminary power analysis determined three replicates for each experimental condition as appropriate, considering the low dispersion of the obtained values. Moreover, the use of triplicate samples is an accepted 'rule of the thumb' when sustained by a careful evaluation of characteristics and costs of the experimental design.[31] The mean $\log_{10}$ reduction was calculated for the tested samples on day 3 and day 14 relative to the initial concentration on day 0 according to Zelver *et al*[32] using Microsoft Excel V.2010. Mann-Whitney U test was employed to evaluate the $\log_{10}$ reduction by Kerasave against each test organism compared with control on day 3 and day 14. A p value less than or equal to 0.05 was considered statistically significant.

RESULTS

Table 1 presents the mean concentration (CFU/mL) and the $\log_{10}$ decrease of all tested strains obtained in growth controls and Kerasave after inoculation with approximately 10^5–10^6 CFU; the time-kill curves are presented in figure 2.

Kerasave reduced the concentration of all strains after 3 days of incubation at 2°C–8°C (table 1 and figure 2). After 3 days, the highest $\log_{10}$ decrease values were observed for *P. aeruginosa* and *K. pneumoniæ*, whereas the lowest values were observed for *A. brasiliensis*, *B. subtilis* and *F. solani* (table 1). After 3 and 14 days of incubation at 2°C–8°C, differences in the $\log_{10}$ decrease between Kerasave and controls were statistically significant according to Mann-Whitney U test (p<0.01; table 1) for all tested strains, except for *A. brasiliensis* (p=0.6396, Mann-Whitney U test), for which a negative $\log_{10}$ decrease was obtained (table 1), corresponding to microbial growth.

After 14 days in Kerasave, the microbial concentration further decreased for all strains, except for *B. subtilis*, whose extent remained unchanged, *K. pneumoniæ*, which was eradicated on day 3, and *A. brasiliensis*, which showed microbial growth (table 1 and figure 2).

Overall, the microbial concentration remained almost unvaried for all strains in the growth controls, except for *A. brasiliensis*, *E. faecalis* and *E. cloacae*, for which evident growth was observed after 14 days (table 1 and figure 2).

The validation study conducted on RESEP showed effective elimination of the antibiotics from Kerasave and microbial recovery after 45 min of treatment with RESEP at RT (online supplemental tables 1 and 2), thus complying with the acceptability criteria adopted.

DISCUSSION

Kerasave is a newly developed medium intended for storage of human donor corneas at 2°C–8°C for up to 14 days. This medium has the peculiarity of being supplied with an antifungal amphotericin B tablet.[18]

Unlike our previous study, in which only *Candida* spp were tested at shorter time intervals,[17 18] the present study investigated nine micro-organisms incubated in Kerasave up to 14 days to simulate the eye bank common practice and cover the intended use of the device. Indeed,

Table 1 Concentration (CFU/mL) and $\log_{10}$ decrease obtained in the time-kill studies of Kerasave and growth controls for each strain

Strain and incubation time (days)	Mean concentration (CFU/mL)		Log$_{10}$ decrease		
	Growth controls	Kerasave	Growth controls	Kerasave	P value*
Candida albicans ATCC 10231					
0	$4.45 \times 10^5 \pm 8.50 \times 10^4$	$4.45 \times 10^5 \pm 8.50 \times 10^4$			
3	$2.68 \times 10^5 \pm 4.56 \times 10^4$	$4.83 \times 10^2 \pm 9.65 \times 10^1$	0.18 ± 0.10	2.97 ± 0.11	<0.001
14	$2.93 \times 10^5 \pm 6.02 \times 10^4$	$0.18 \times 10^0 \pm 0.12 \times 10^0$	0.19 ± 0.11	5.52 ± 0.08	<0.001
Fusarium solani DSM-N°1164					
0	$3.15 \times 10^5 \pm 9.57 \times 10^3$	$3.15 \times 10^5 \pm 9.57 \times 10^3$			
3	$2.35 \times 10^5 \pm 2.09 \times 10^4$	$6.15 \times 10^4 \pm 3.06 \times 10^3$	0.13 ± 0.04	0.67 ± 0.03	0.004
14	$2.12 \times 10^5 \pm 2.65 \times 10^4$	$1.62 \times 10^4 \pm 1.07 \times 10^3$	0.19 ± 0.05	1.25 ± 0.03	0.006
Aspergillus brasiliensis ATCC 16404					
0	$4.45 \times 10^5 \pm 6.58 \times 10^4$	$4.45 \times 10^5 \pm 6.58 \times 10^4$			
3	$1.58 \times 10^6 \pm 3.89 \times 10^5$	$2.90 \times 10^5 \pm 6.41 \times 10^4$	-0.44 ± 0.13	0.30 ± 0.14	<0.001
14	$2.03 \times 10^7 \pm 3.80 \times 10^6$	$2.29 \times 10^7 \pm 5.07 \times 10^6$	-1.64 ± 0.10	-1.62 ± 0.11	0.640
Staphylococcus aureus ATCC 6538					
0	$4.86 \times 10^5 \pm 5.86 \times 10^4$	$4.86 \times 10^5 \pm 5.86 \times 10^4$			
3	$8.03 \times 10^5 \pm 1.50 \times 10^5$	$3.61 \times 10^3 \pm 7.55 \times 10^2$	-0.13 ± 0.12	2.27 ± 0.11	<0.001
14	$2.27 \times 10^6 \pm 4.99 \times 10^5$	$1.84 \times 10^2 \pm 2.39 \times 10^1$	-0.60 ± 0.11	3.39 ± 0.07	<0.001
Bacillus subtilis spizizenii ATCC 6633					
0	$2.13 \times 10^6 \pm 2.93 \times 10^5$	$2.13 \times 10^6 \pm 2.93 \times 10^5$			
3	$1.44 \times 10^7 \pm 4.95 \times 10^6$	$1.01 \times 10^6 \pm 1.22 \times 10^5$	-0.51 ± 0.14	0.25 ± 0.08	<0.001
14	$3.38 \times 10^6 \pm 4.60 \times 10^5$	$1.19 \times 10^6 \pm 1.30 \times 10^5$	-0.21 ± 0.09	0.18 ± 0.08	0.001
Enterococcus faecalis ATCC 29212					
0	$4.07 \times 10^6 \pm 4.04 \times 10^5$	$4.07 \times 10^6 \pm 4.04 \times 10^5$			
3	$1.93 \times 10^7 \pm 1.04 \times 10^6$	$3.11 \times 10^4 \pm 4.72 \times 10^3$	-0.76 ± 0.06	2.11 ± 0.09	<0.001
14	$3.25 \times 10^{10} \pm 5.78 \times 10^9$	$1.20 \times 10^3 \pm 1.78 \times 10^2$	-3.91 ± 0.11	3.53 ± 0.08	<0.001
Pseudomonas aeruginosa ATCC 9027					
0	$2.10 \times 10^6 \pm 3.11 \times 10^5$	$2.10 \times 10^6 \pm 3.11 \times 10^5$			
3	$1.11 \times 10^6 \pm 2.88 \times 10^5$	$1.73 \times 10^2 \pm 3.20 \times 10^1$	0.47 ± 0.14	4.15 ± 0.10	<0.001
14	$3.51 \times 10^6 \pm 7.97 \times 10^5$	$9.78 \times 10^{-1} \pm 1.78 \times 10^{-1}$	-0.09 ± 0.12	6.20 ± 0.08	<0.001
Klebsiella pneumoniae ATCC 10031					
0	$2.72 \times 10^5 \pm 2.82 \times 10^4$	$2.72 \times 10^5 \pm 2.82 \times 10^4$			
3	$2.77 \times 10^5 \pm 3.70 \times 10^4$	$0.00 \times 10^0 \pm 0.00 \times 10^0$	-0.03 ± 0.06	5.37 ± 0.04	<0.001
14	$1.83 \times 10^5 \pm 3.64 \times 10^4$	$0.00 \times 10^0 \pm 0.00 \times 10^0$	0.19 ± 0.07	5.37 ± 0.04	<0.001
Enterobacter cloacae ATCC 35030					
0	$3.92 \times 10^5 \pm 5.24 \times 10^4$	$3.92 \times 10^5 \pm 5.24 \times 10^4$			
3	$2.35 \times 10^5 \pm 3.23 \times 10^4$	$3.95 \times 10^3 \pm 1.76 \times 10^3$	0.16 ± 0.08	2.67 ± 0.27	<0.001
14	$8.20 \times 10^8 \pm 5.87 \times 10^7$	$0.00 \times 10^0 \pm 0.00 \times 10^0$	-3.42 ± 0.06	5.49 ± 0.05	<0.001

Data are expressed as mean±SEM.
*P value corresponds to Mann-Whitney U test on the $\log_{10}$ decrease values between Kerasave and growth controls.
ATCC, American Type Culture Collection; CFU, colony forming unit; DSM, Deutsche Sammlung von Mikroorganismen.

the release of the cornea for transplantation usually does not exceed 7 days of storage at 4°C,[2] and the maximum time of use of the device indicated by the manufacturer corresponds to 14 days. Therefore, in the present study, data were not acquired beyond 14 days. All the tested micro-organisms showed significantly lower microbial concentrations in Kerasave than in the growth controls of all the tested time points, except for *A. brasiliensis* on day 14, which was comparable with the control. Our findings indicated that the medium effectively eliminated
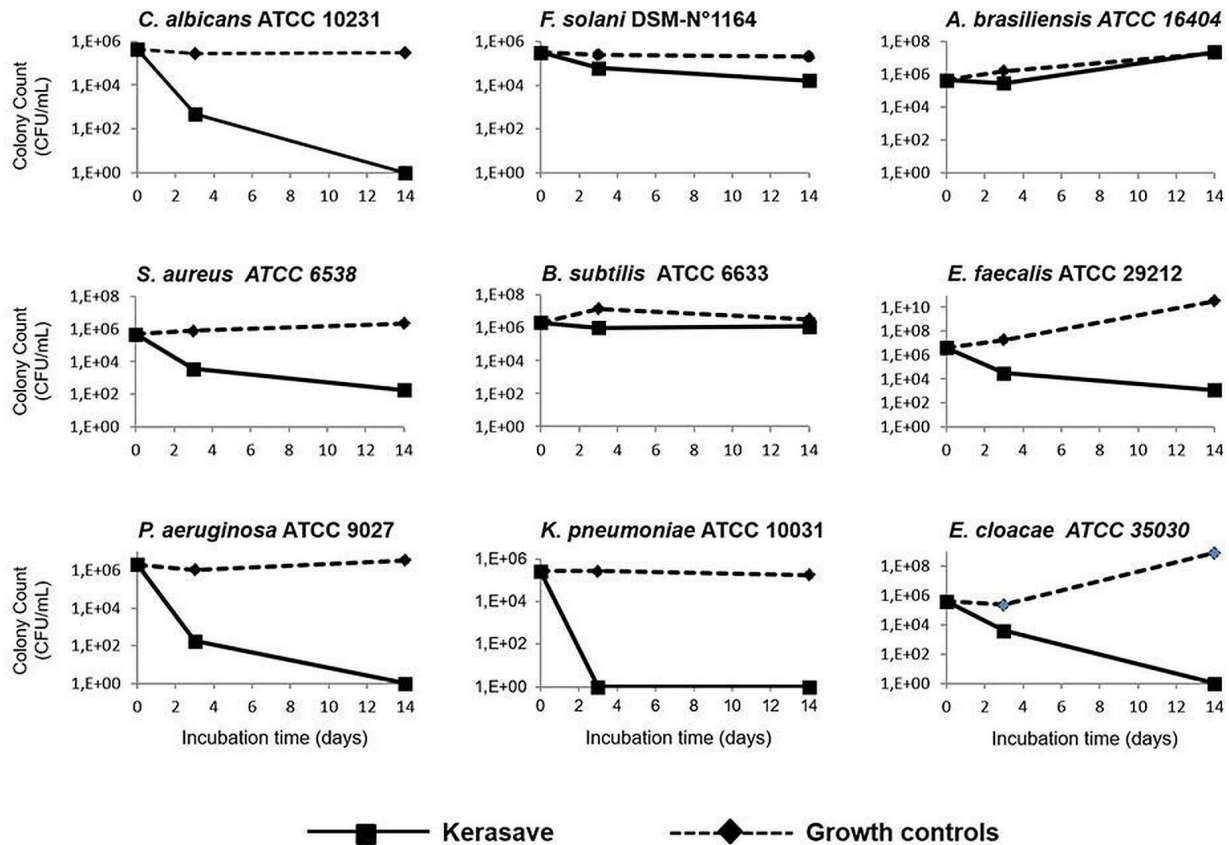
Figure 2 Time-kill curves. The graphs show the mean values of microbial concentration, expressed as CFU/mL, observed in the Kerasave samples and growth controls on day 0, day 3 and day 14 for each tested micro-organism. CFU, colony forming unit. ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen.

seven out of nine (78%) tested micro-organisms. This was accomplished despite the low storage temperature of 4°C, which is considered to be a condition resulting in poor antimicrobial efficacy.[11 33]

The bactericidal activity is achieved when a 3 $\log_{10}$ decrease is obtained.[34–36] In the time-kill assay, a very high initial microbial concentration inoculum (10^5–10^6 CFU/mL) is used to allow quantification of the killing efficacy. The high inoculum concentration used in our study does not reflect a clinical situation as it is significantly higher than the bacterial concentration observed in the blood of patients with sepsis (10^2–10^3 CFU/mL)[37]; therefore, complete elimination of the tested micro-organism is not expected in these assays. In our study, the 3 $\log_{10}$ decrease indicates bactericidal activity of Kerasave for some micro-organisms. Complete microbial elimination (5–6 $\log_{10}$ reduction) would correspond to the performance of disinfectants and sterilisation processes, which is not expected to be achieved by Kerasave.

A $\log_{10}$ reduction ≥3 was achieved after 3 days of incubation in Kerasave for *C. albicans*, *P. aeruginosa* and *K. pneumoniae* strains, and was achieved on day 14 for *S. aureus*, *E. faecalis* and *E. cloacae*. The elimination of *C. albicans* was in line with the results of our previous study, in which *C. albicans* killing was assessed after 10 days of incubation.[18] In this study, Kerasave was shown

to be effective during the 14 days of storage. For three strains (*A. brasiliensis*, *F. solani* and *B. subtilis*), a low or negative $\log_{10}$ decrease was observed, which indicated low microbial elimination or slight microbial growth over time. For both *A. brasiliensis* and *F. solani*, which are filamentous fungi, a low killing efficacy of 2.5 μg/mL amphotericin B contained in Kerasave was expected as the minimal inhibition concentration values for amphotericin B reported in the literature were 0.03–4 μg/mL and 4–8 μg/mL for *A. brasiliensis* and *F. solani*, respectively.[38–41] In a previous study, Duncan *et al*[42] found that the supplementation of the hypothermic corneal storage medium with amphotericin B at 0.25 μg/mL effectively reduced the growth of *F. solani*.

Along with the lower concentration of amphotericin B, there are some other differences in the experimental conditions between this and our study, that is, a lower concentration of the inoculum (2.5×10^3 CFU/mL vs 10^5–10^6 CFU/mL) and a lower extent of growth reduction. Nevertheless, the authors also concluded that amphotericin B appeared to be a reasonable candidate drug for an antifungal additive to a corneal storage medium. In a recent study, Kaymar *et al*[43] found that amphotericin B at 2.5 μg/mL was ineffective in eliminating small loads of *Candida* and *Fusarium* species (up to 10^3 CFU/mL). However, the goal of that study was to achieve complete elimination of fungi limited

to single testing due to the scarcity of corneal tissue. Unlike our study, the growth reduction was not quantified. Thus, the differences in the experimental design might explain why the authors concluded that the eradication of fungal contamination of donor corneal tissue might require complementary approaches of antifungal supplementation of hypothermic storage media. However, based on the results of Kapur *et al*'s study,[44] which examined the effect of the hypothermic corneal storage medium with and without tissue growth of similar species, it appears that the presence of corneal tissue does not affect viable counts.

The concentration of amphotericin B in Kerasave represents the optimal balance between the efficacy and safety for preserving the donor cornea[17] and was selected under the intended use of Kerasave, which corresponds to corneal storage rather than corneal disinfection. Although corneal disinfection as intended for disinfectants (povidone-iodine solutions) is out of Kerasave's intended use,[18] Kerasave temporarily prevented the growth of *F. solani*, for which a moderate 1.25 $\log_{10}$ decrease was observed after 14 days. In any case, we can consider Kerasave effective in decreasing or eliminating contamination from *Candida* spp, which is the most commonly encountered pathogen.[7] Other fungi were not commonly encountered in donor corneal transplantation.[23 24 45]

Surprisingly, a very low $\log_{10}$ decrease of 0.18 was observed for *B. subtilis* after 14 days in Kerasave, indicating a bacteriostatic rather than bactericidal effect for this strain under the tested conditions. Data in the literature reported *B. subtilis* to be sensitive to gentamicin (4.0 mg/L; 0.125 µg/mL) and resistant to streptomycin[45–47]; the gentamicin content of Kerasave, which is significantly higher than that reported in the literature, led us to expect higher killing efficacy of *B. subtilis spizizenii* by Kerasave. The low killing efficacy was confirmed when we repeated the time-kill assay at 4°C and 31°C after 3 days of incubation to evaluate whether the incubation temperature could influence the killing efficacy of Kerasave (data not shown). At 31°C, a 0.6 $\log_{10}$ decrease was observed in Kerasave after 3 days, whereas marked growth was observed in controls (−2.8 $\log_{10}$ decrease; data not shown). We thus confirmed the bacteriostatic effect of Kerasave for *B. subtilis* under tested conditions. Moreover, we hypothesised that *B. subtilis spizizenii* can turn into sporulation, enabling survival under adverse conditions.[48 49] However, microscopy studies to check for possible *B. subtilis spizizenii* sporulation were not conducted as they were beyond the scope of the present study.

With regard to growth controls, most strains maintained the initial concentration or showed growth along with incubation (*A. brasiliensis*, *E. faecalis* and *E. cloacae*). Previous studies[15 42] compared Kerasave with other corneal storage media. Contrarily, in this study, we chose to use growth media as control samples to confirm microbial growth of the strains under tested conditions and not in comparison with other corneal storage media since the validity of the time-kill assay and evaluation of the recovery of viable micro-organisms require optimal microbial growth and absence of any factors that would interfere with it. As discussed by Tran *et al*,[17] approaches that do not use a step to neutralise the antimicrobials present in the culture samples used for the quantification of the micro-organisms can lead to the presence of some residual amounts of drugs, which can prevent growth on culture plates.[17] Kerasave contains different antimicrobials, and their interference with microbial growth is predictable. Therefore, to avoid false-negative results, RESEP was used before plating the Kerasave samples on agar plates. The treatment of Kerasave samples with RESEP was validated prior to its use in the time-kill assay using the ultra-high-performance liquid chromatography which revealed total removal of antimicrobials from Kerasave and complete recovery of viable micro-organisms was obtained in RESEP-treated or untreated samples (online supplemental tables 1 and 2).

The RESEP treatment was not performed on *F. solani* containing samples since growth inhibition of *F. solani* by Kerasave was not observed. Thus, the dilution plate technique was sufficient to obtain a reliable result in the time-kill study.

Safety study of Kerasave was out of the scope of the present study as it has been previously investigated in cells via a cytotoxicity test, biocompatibility animal studies according to International Organization for Standardization standards[17 18] and in donor corneas.[15 45]

This study completes the data obtained in our previous work on the efficacy of the Kerasave medium, confirming the killing of the most common corneal contaminants by Kerasave under hypothermic corneal storage conditions even after a short incubation time (*C. albicans*, *P. aeruginosa* and *K. pneumoniae*) and preventing the microbial growth of all the other tested strains. In this study, the killing efficacy of Kerasave was directly related to the time of incubation, as observed for *S. aureus*, *E. faecalis* and *E. cloacae*. The killing efficacy of Kerasave could be limited in case of contaminations by micro-organisms resistant to the antimicrobials present in Kerasave (amphotericin B, streptomycin sulfate and gentamicin sulfate), and eye banks should consider that a short storage interval and storage temperature of 4°C could prevent the eradication of high contamination from donor tissues.[18] Finally, the hypothermic corneal storage medium Kerasave is a promising tool for enhancing the safety of the recipient of the transplanted cornea.

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REFERENCES

1 Reinhart WJ, Musch DC, Jacobs DS, *et al*. Deep anterior lamellar keratoplasty as an alternative to penetrating keratoplasty a report by the American Academy of ophthalmology. *Ophthalmology* 2011;118:209–18.
2 Armitage WJ. Preservation of human cornea. *Transfus Med Hemother* 2011;38:143–7.
3 Tan DTH, Dart JKG, Holland EJ, *et al*. Corneal transplantation. *Lancet* 2012;379:1749–61.
4 Rehany U, Balut G, Lefler E, *et al*. The prevalence and risk factors for donor corneal button contamination and its association with ocular infection after transplantation. *Cornea* 2004;23:649–54.
5 Chen JY, Jones MN, Srinivasan S, *et al*. Endophthalmitis after penetrating keratoplasty. *Ophthalmology* 2015;122:25–30.
6 Aldave AJ, DeMatteo J, Glasser DB, *et al*. Report of the eye bank association of America medical Advisory board Subcommittee on fungal infection after corneal transplantation. *Cornea* 2013;32:149–54.
7 Thareja T, Kowalski R, Kamyar R, *et al*. Fungal infection after keratoplasty and the role of antifungal supplementation to storage solution: a review. *Br J Ophthalmol* 2020;104:1–4.
8 Fontana L, Moramarco A, Mandarà E, *et al*. Interface infectious keratitis after anterior and posterior lamellar keratoplasty. clinical features and treatment strategies. A review. *Br J Ophthalmol* 2019;103:307–14.
9 Palioura S, Sivaraman K, Joag M, *et al*. *Candida* endophthalmitis after Descemet stripping automated endothelial keratoplasty with grafts from both eyes of a donor with possible systemic candidiasis. *Cornea* 2018;37:515–8.
10 Brothers KM, Shanks RMQ, Hurlbert S, *et al*. Association between fungal contamination and eye bank–prepared endothelial keratoplasty tissue: temperature-dependent risk factors and antifungal supplementation of Optisol–gentamicin and streptomycin. *JAMA Ophthalmol* 2017;135:1184–90.
11 Pels E, Beele H, Claerhout I. Eye bank issues: II. Preservation techniques: warm versus cold storage. *Int Ophthalmol* 2008;28:155–63.
12 Hassan SS, Wilhelmus KR, Medical Review Subcommittee of the Eye Bank Association of America. Eye-banking risk factors for fungal endophthalmitis compared with bacterial endophthalmitis after corneal transplantation. *Am J Ophthalmol* 2005;139:685–90.
13 Duncan K, Parker J, Hoover C, *et al*. The effect of light exposure on the efficacy and safety of amphotericin B in corneal storage media. *JAMA Ophthalmol* 2016;134:432–6.
14 Layer N, Cevallos V, Maxwell AJ, *et al*. Efficacy and safety of antifungal additives in Optisol-GS corneal storage medium. *JAMA Ophthalmol* 2014;132:832–7.
15 Mistò R, Giurgola L, Pateri F, *et al*. A new storage medium containing amphotericin B versus Optisol-GS for preservation of human donor corneas. *Br J Ophthalmol* 2020;0. doi:10.1136/bjophthalmol-2020-317136. [Epub ahead of print: 10 Nov 2020].
16 Doshi H, Pabon S, Price MO, *et al*. Overview of systemic *Candida* infections in hospital settings and report of candida after DMEK successfully treated with antifungals and partial graft excision. *Cornea* 2018;37:1071–4.
17 Tran KD, Aldrich BT, D'Amato Tóthová J, *et al*. Efficacy and safety of various amphotericin B concentrations on *Candida albicans* in cold storage conditions. *Cornea* 2020;39:110–7.
18 Giurgola L, Gatto C, Parel J-M, *et al*. Antimycotic efficacy and safety of a new cold corneal storage medium by time-kill and toxicity studies. *Cornea* 2019;38:1314–21.
19 Escapini H, Olson RJ, Kaufman HE. Donor cornea contamination with McCarey-Kaufman medium preservation. *Am J Ophthalmol* 1979;88:59–62.
20 Poole TG, Insler MS. Contamination of donor cornea by gentamicin-resistant organisms. *Am J Ophthalmol* 1984;97:560–4.
21 Pardos GJ, Gallagher MA. Microbial contamination of donor eyes. *Arch Ophthal* 1982;100:1611–3.
22 Albon J, Armstrong M, Tullo AB. Bacterial contamination of human organ-cultured corneas. *Cornea* 2001;20:260–3.
23 Broniek G, Langwińska-Wośko E, Sybilska M, *et al*. Prevalence of bacteria and fungi in samples of cornea preservation fluid. *Arch Med Sci* 2018;14:541–6.
24 Fontana L, Errani PG, Zerbinati A, *et al*. Frequency of positive donor rim cultures after penetrating keratoplasty using hypothermic and organ-cultured donor corneas. *Cornea* 2007;26:552–6.
25 Zanetti E, Bruni A, Mucignat G, *et al*. Bacterial contamination of human organ-cultured corneas. *Cornea* 2005;24:603–7.
26 Buzzi M, Guarino A, Gatto C, *et al*. Residual antibiotics in decontaminated human cardiovascular tissues intended for transplantation and risk of falsely negative microbiological analyses. *PLoS One* 2014;9:e112679.
27 Mistò R, Giurgola L, Pateri F, *et al*. Method for sterility testing of corneal storage and transport media after removal of interfering antimicrobials: prospective validation study in compliance with the European Pharmacopoeia. *BMJ Open Ophthalmol* 2017;2:e000093.
28 Vignola R, Mistò R, Giurgola L, *et al*. A two-centre validation study of sterility test of corneal storage media with elimination of interfering antimicrobials in compliance with the European Pharmacopoeia. *Cell Tissue Bank* 2019;20:275–85.
29 Skenderi Z, Giurgola L, Gatto C, *et al*. Increased sensitivity of microbiological testing of cornea organ culture medium by additional resin treatment. *BMJ Open Ophthalmol* 2018;3:e000173.
30 Cohen J. *Statistical power analysis for the behavioral sciences*. Hillsdale, NJ: Erlbaum, 1988.
31 Singer JM, Pedroso-de-Lima AC, Tanaka NI, *et al*. To triplicate or not to triplicate? *Chemometrics and Intelligent Laboratory Systems* 2007;86:82–5.
32 Zelver N, Hamilton M, Goeres D, *et al*. Development of a standardized antibiofilm test. *Methods Enzymol* 2001;337:363–76.
33 Pitt TL, Tidey K, Roy A, *et al*. Activity of four antimicrobial cocktails for tissue allograft decontamination against bacteria and Candida *spp*. of known susceptibility at different temperatures. *Cell Tissue Bank* 2014;15:119–25.
34 Leite MCA, Bezerra APdeB, de Sousa JP, *et al*. Evaluation of antifungal activity and mechanism of action of citral against *Candida albicans*. *Evid Based Complement Alternat Med* 2014;2014:378280.
35 Pfaller MA, Sheehan DJ, Rex JH. Determination of fungicidal activities against yeasts and molds: lessons learned from bactericidal testing and the need for standardization. *Clin Microbiol Rev* 2004;17:268–80.
36 Van Dijck P, Sjollema J, Cammue BP, *et al*. Methodologies for *in vitro* and *in vivo* evaluation of efficacy of antifungal and antibiofilm agents and surface coatings against fungal biofilms. *Microb Cell* 2018;5:300–26.
37 Yagupsky P, Nolte FS. Quantitative aspects of septicemia. *Clin Microbiol Rev* 1990;3:269–79.
38 Tokarzewski S, Ziółkowska G, Nowakiewicz A. Susceptibility testing of *Aspergillus niger* strains isolated from poultry to antifungal drugs--a comparative study of the disk diffusion, broth microdilution (M 38-A) and Etest methods. *Pol J Vet Sci* 2012;15:125–33.
39 Borman AM, Fraser M, Palmer MD, *et al*. MIC distributions and evaluation of fungicidal activity for amphotericin B, itraconazole, voriconazole, posaconazole and caspofungin and 20 species of

pathogenic filamentous fungi determined using the CLSI broth microdilution method. *J Fungi* 2017;3:27.

40 Lass-Flörl C, Mayr A, Perkhofer S, *et al*. Activities of antifungal agents against yeasts and filamentous fungi: assessment according to the methodology of the European Committee on antimicrobial susceptibility testing. *Antimicrob Agents Chemother* 2008;52:3637–41.

41 Lalitha P, Shapiro BL, Srinivasan M, *et al*. Antimicrobial susceptibility of *Fusarium*, *Aspergillus*, and other filamentous fungi isolated from keratitis. *Arch Ophthalmol* 2007;125:789–93.

42 Duncan K, Parker J, Hoover C, *et al*. Efficacy of amphotericin B against Fusarium and Aspergillus in corneal storage medium. *Eye Contact Lens* 2018;44:390–2.

43 Kaymar RM, Shatten JD D, *et al*. Washing to dilute may be the answer to eliminate fungus from donor corneal tissue. *Int J Eye Banking* 2020;8.

44 Kapur R, Tu EY, Pendland SL, *et al*. The effect of temperature on the antimicrobial activity of Optisol-GS. *Cornea* 2006;25:319–24.

45 Perry I, Peterson K, Tóthová Jana D'Amato, *et al*. Performance of new hypothermic corneal storage media with an antimycotic tablet

in comparison to traditional hypothermic media during simulated eye bank processing. *Cornea* 2020;39:1031–9.

46 Adimpong DB, Sørensen KI, Thorsen L, *et al*. Antimicrobial susceptibility of *Bacillus* strains isolated from primary starters for African traditional bread production and characterization of the bacitracin operon and bacitracin biosynthesis. *Appl Environ Microbiol* 2012;78:7903–14.

47 Lin JT, Connelly MB, Amolo C, *et al*. Global transcriptional response of *Bacillus subtilis* to treatment with subinhibitory concentrations of antibiotics that inhibit protein synthesis. *Antimicrob Agents Chemother* 2005;49:1915–26.

48 Budde I, Steil L, Scharf C, *et al*. Adaptation of *Bacillus subtilis* to growth at low temperature: a combined transcriptomic and proteomic appraisal. *Microbiology* 2006;152:831–53.

49 Jabbari S, Heap JT, King JR. Mathematical modelling of the sporulation-initiation network in *Bacillus subtilis* revealing the dual role of the putative quorum-sensing signal molecule PhrA. *Bull Math Biol* 2011;73:181–211.