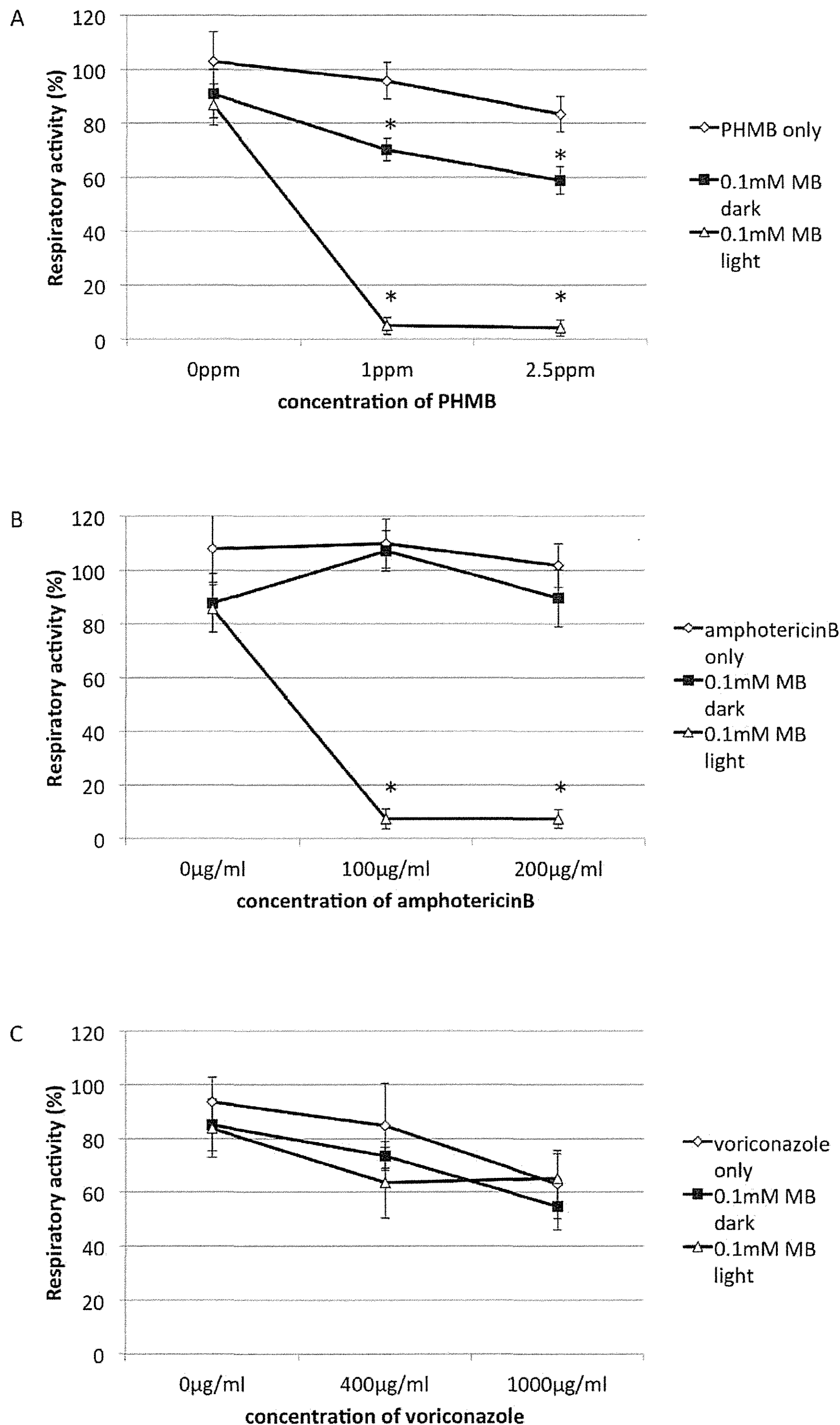

FIGURE 4. Combined effect of MB-PDT (incubation in 0.1 mM MB and irradiation at a dose of 10.8 J/cm^2) and antimicrobial drug pretreatment at various concentrations. PHMB (**A**) and amphotericin B (**B**) were found to have a synergistic effect with MB-PDT, whereas MB-PDT and voriconazole (**C**) did not show a synergistic effect compared with the antimicrobial drug control $P < 0.05$. $^*P < 0.05$.

acanthamoeba respiratory activity is dependent on the dose of radiation. In previous reports, PDT has been found to have a similar radiation dose-dependent effect on other pathogens besides acanthamoeba.[17-20] In other words, the efficacy of PDT is thought to increase in proportion to light intensity and exposure time.

We found that conventionally used medications and MB-PDT have an additive or synergistic effect against trophozoites. This suggests that MB-PDT could be a valuable adjuvant to antimicrobial therapy in AK cases that show little or no improvement with conventional antiamoebal treatment. Both PHMB and amphotericin B produced a particularly notable synergistic effect with MB-PDT against trophozoites. PHMB induces changes in cell membrane permeability, leading to potassium efflux and eventual loss of membrane function and cell death.[41] As with other polyene antifungals, amphotericin B

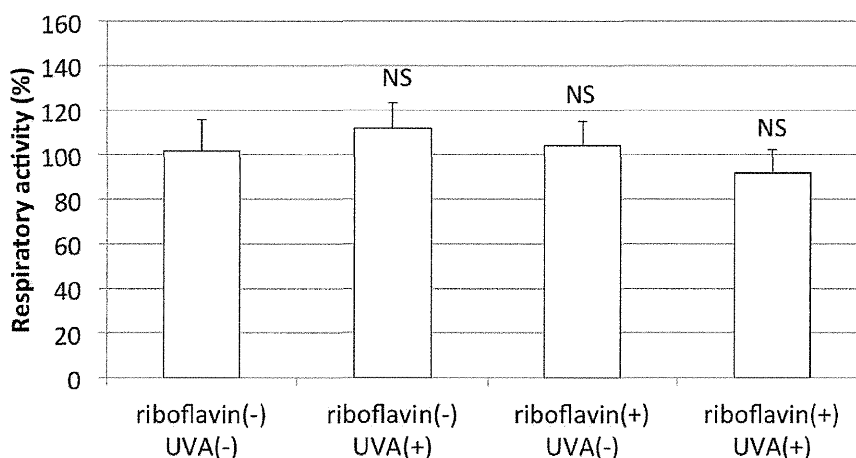

FIGURE 5. Comparison of the effect of four regimens of riboflavin UVA cross-linking treatment determined by the CTC biocidal assay. Trophozoites were incubated with 0.1% riboflavin for 1 hour, then washed with PBS three times and irradiated with 365 nm UVA light at 10.8 J/cm^2. A value of 100% was assigned to the respiratory activity of untreated control cells (PBS). No significant difference in respiratory activity was found between any cross-linking group and the nonirradiated PBS control group ($P > 0.05$). Data shown are the means and SEM from a representative experiment ($n = 5$/experimental condition) that was repeated five times with similar results.

is believed to interact with membrane sterols and produce an aggregate that forms a transmembrane channel. The synergistic effects of MB and medication are brought about because MB can leak into the cell more quickly after prior damage to the cell membrane.

In contrast, voriconazole requires a longer period of time to exert a membrane-damaging effect compared with PHMB and amphotericin B,[42] and thus combinations involving voriconazole did not show a synergistic effect. Since AK treatment generally takes a long time, medication toxicity can occasionally become a problem. In situations where two medications exert a synergistic effect, toxicity can be avoided by lowering each medication dosage below the concentration usually used in single-agent therapy. In addition, it has been reported that organisms can easily become resistant to low-dose single-drug antiamoebic therapy,[43] so by using multiple-agent therapy, the emergence of resistance can be prevented.

Cross-linking induced by riboflavin-UVA combination therapy, which is often used to treat keratoconus, works similarly to MB-PDT by producing reactive oxygen species. Recently, a series of reports have been published regarding the promising clinical applications of cross-linking for corneal infections.[10,44] Khan et al.[45] contend that cross-linking is useful for AK. However, our in vitro experimental results suggest that cross-linking is markedly less effective than MB-PDT against trophozoites. Similarly, Del Buey et al.[46] concluded that a single dose (30 or 60 minutes) of cross-linking cannot achieve eradication of the two different *Acanthamoeba* strains examined. However, their evaluation method did not quantify reductions in the population of viable amoeba; it detected only the presence of amoeba, and their growth and movement in the agar media. In this respect, the CTC biocidal assay is advantageous because it enables quantitative comparison. Our study using the CTC biocidal assay shows that MB-PDT is more effective than riboflavin-UVA combination therapy.

We suggest that MB-PDT has the potential to provide a valuable adjuvant to antimicrobial therapy in AK when combined with antiamoebal agents. Since the cornea is an organ that is exposed to the outside world, local administration of medication and irradiation can be carried out easily, and for this reason, AK therapy appears to be a feasible application of PDT. Furthermore, we examined the toxicity of PDT treatment in vivo using C57BL/6 mouse corneas with epithelial defects, and found that MB-PDT did not cause any appreciable damage to the cornea (see Supplementary Fig. S1; link to supplemental material: http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9828/-/DCSupplemental). Thus, we conclude that PDT treatment would produce minimal toxicity in vivo. Although our results show that MB-PDT is efficacious in vitro, these results may not correlate with in vivo efficacy; therefore, further animal studies are under way to test the in vivo efficacy of this treatment for AK.

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References

1. Illingworth CD, Cook SD. Acanthamoeba keratitis. *Surv Ophthalmol.* 1998;42:493-508.
2. Dart JK, Saw VP, Kilvington S. Acanthamoeba keratitis: diagnosis and treatment update 2009. *Am J Ophthalmol.* 2009;148:487-499.
3. Qian Y, Meisler DM, Langston RH, Jeng BH. Clinical experience with Acanthamoeba keratitis at the Cole Eye Institute, 1999-2008. *Cornea.* 2010;29:1016-1021.
4. Duguid IG, Dart JK, Morlet N, et al. Outcome of acanthamoeba keratitis treated with polyhexamethyl biguanide and propamidine. *Ophthalmology.* 1997;104:1587-1592.
5. Hargrave SL, McCulley JP, Husseini Z. Results of a trial of combined propamidene isethionate and neomycin therapy for Acanthamoeba keratitis. Brolene Study Group. *Ophthalmology.* 1999;106:952-957.
6. Butler TK, Males JJ, Robinson LP, et al. Six-year review of Acanthamoeba keratitis in New South Wales, Australia: 1997-2002. *Clin Exp Ophthalmol.* 2005;33:41-46.
7. Lim N, Goh D, Bunce C, et al. Comparison of polyhexamethylene biguanide and chlorhexidine as monotherapy agents in the treatment of Acanthamoeba keratitis. *Am J Ophthalmol.* 2008;145:130-135.
8. Iseri HP, Thiel MA, Hafezi F, Kampmeier J, Seiler T. Ultraviolet A/riboflavin corneal cross-linking for infectious keratitis associated with corneal melts. *Cornea.* 2008;27:590-594.

9. Moren H, Malmsjo M, Mortensen J, Ohrstrom A. Riboflavin and ultraviolet A collagen crosslinking of the cornea for the treatment of keratitis. *Cornea*. 2010;29:102–104.
10. Makdoumi K, Mortensen J, Crafoord S. Infectious keratitis treated with corneal crosslinking. *Cornea*. 2010;29:1353–1358.
11. Wainwright M. Photodynamic antimicrobial chemotherapy (PACT). *J Antimicrob Chemother*. 1998;42:13–28.
12. Hamblin MR, Hasan T. Photodynamic therapy: a new antimicrobial approach to infectious disease? *Photochem Photobiol Sci*. 2004;3:436–450.
13. Jori G, Fabris C, Soncin M, et al. Photodynamic therapy in the treatment of microbial infections: basic principles and perspective applications. *Lasers Surg Med*. 2006;38:468–481.
14. Donnelly RF, McCarron PA, Tunney MM. Antifungal photodynamic therapy. *Microbiol Res*. 2008;163:1–12.
15. Tardivo JP, Giglio AD, Oliveira CS, et al. Methylene blue in photodynamic therapy: from basic mechanisms to clinical applications. *Photodiagnosis Photodyn Ther*. 2005;2:175–191.
16. Wainwright M. The development of phenothiazinium photosensitisers. *Photodiagnosis Photodyn Ther*. 2005;2:263–272.
17. Zeina B, Greenman J, Purcell WM, Das B. Killing of cutaneous microbial species by photodynamic therapy. *Br J Dermatol*. 2001;144:274–278.
18. Ragas X, Dai T, Tegos GP, et al. Photodynamic inactivation of Acinetobacter baumannii using phenothiazinium dyes: in vitro and in vivo studies. *Lasers Surg Med*. 2010;42:384–390.
19. Shih MH, Huang FC. Effects of photodynamic therapy on rapidly growing nontuberculous mycobacteria keratitis. *Invest Ophthalmol Vis Sci*. 2011;52:223–229.
20. Peloi LS, Soares RR, Biondo CE, et al. Photodynamic effect of light-emitting diode light on cell growth inhibition induced by methylene blue. *J Biosci*. 2008;33:231–237.
21. Giroldo LM, Felipe MP, de Oliveira MA, et al. Photodynamic antimicrobial chemotherapy (PACT) with methylene blue increases membrane permeability in Candida albicans. *Lasers Med Sci*. 2009;24:109–112.
22. Neff R, Ray S, Benton W, Wilborn M. Induction of synchronous encystment (differentiation) in Acanthamoeba sp. *Methods Cell Physiol*. 1964;1:55–83.
23. Kobayashi T, Mito T, Watanabe N, et al. Use of 5-cyano-2, 3-tetrazolium chloride (CTC) staining as indicator of biocidal activity in rapid assay for anti-*Acanthamoeba* agents. *J Clin Microbiol*. 2012;50:1606–1612.
24. Morton LD, Mclaughlin GL, Whiteley HE. Effects of temperature, amebic strain, and carbohydrates on Acanthamoeba adherence to corneal epithelium in vitro. *Infect Immun*. 1991;59:3819–3822.
25. Ondarza RN, Iturbe A, Hernandez E. In vitro antiproliferative effects of neuroleptics, antimycotics and antibiotics on the human pathogens Acanthamoeba polyphaga and Naegleria fowleri. *Arch Med Res*. 2006;3:723–729.
26. Kobayashi T, Gibbon L, Mito T, et al. Efficacy of commercial soft contact lens disinfectant solutions against Acanthamoeba. *Jpn J Ophthalmol*. 2011;55:547–557.
27. Hamilton M, Russo R, Thurston R. Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. *Environ Sci Technol*. 1977;11:714–719.
28. Khunkitti W, Hann AC, Lloyd D, Furr JR, Russell AD. Biguanide-induced changes in Acanthamoeba castellanii: an electron microscopic study. *J Appl Microbiol*. 1998;84:53–62.
29. Mogoa E, Bodet C, Legube B, Hechard Y. Acanthamoeba castellanii: cellular changes induced by chlorination. *Exp Parasitol*. 2010;126:97–102.
30. Ferro S, Guidolin L, Tognon G, Jori G, Coppellotti O. Mechanisms involved in the photosensitized inactivation of *Acanthamoeba palestinensis* trophozoites. *J Appl Microbiol*. 2009;107:1615–1623.
31. Ferro S, Coppellotti O, Roncucci G, Ben Amor T, Jori G. Photosensitized inactivation of Acanthamoeba palestinensis in the cystic stage. *J Appl Microbiol*. 2006;101:206–212.
32. Chen Z, Xuguang S, Zhiqun W, Ran L. In vitro amoebacidal activity of photodynamic therapy on *Acanthamoeba*. *Br J Ophthalmol*. 2008;92:1283–1286.
33. Beattie TK, Seal DV, Tomlinson A, McFadyen AK, Grimason AM. Determination of amoebicidal activities of multipurpose contact lens solutions by using a most probable number enumeration technique. *J Clin Microbiol*. 2003;41:2992–3000.
34. Brown G, Frankl D, Phang T. Continuous infusion of methylene blue for septic shock. *Postgrad Med J*. 1996;72:612–614.
35. Chen YW, Lin JS, Fong JH, et al. Use of methylene blue as a diagnostic aid in early detection of oral cancer and precancerous lesions. *Br J Oral Maxillofac Surg*. 2006;45:590–591.
36. DeHoll JD, Shin PA, Angle JF, Steers WD. Alternative approaches to the management of priapism. *Int J Impotence Res*. 1998;10:11–14.
37. Orth K, Ruck A, Stanescu A, Beger HG. Intraluminal treatment of inoperable oesophageal tumours by intralesional photodynamic therapy with methylene blue. *Lancet*. 1995;345:519–520.
38. Sutherland AD, Faragher IG, Frizelle FA. Intradermal injection of methylene blue for the treatment of refractory pruritus ani. *Colorectal Dis*. 2009;11:282–287.
39. Vandoni RE, Cuttat JF, Wicky S, Suter M. CT-guided methylene-blue labelling before thoracoscopic resection of pulmonary nodules. *Eur J Cardiothorac Surg*. 1998;14:265–270.
40. Kessel D, Luo Y. Mitochondrial photodamage and PDT-induced apoptosis. *J Photochem Photobiol B Biol*. 1998;42:89–95.
41. McDonnell G, Russell AD. Antiseptics and disinfectants: activity, action, and resistance. *Clin Microbiol Rev*. 1999;12:147–179.
42. Maertens JA. History of the development of azole derivatives. *Clin Microbiol Infect*. 2004;10:1–10.
43. Hay J, Kirkness CM, Seal DV, Wright P. Drug resistance and Acanthamoeba keratitis: the quest for alternative antiprotozoal chemotherapy. *Eye*. 1994;8:555–563.
44. Martins SA, Combs JC, Noguera G, et al. Antimicrobial efficacy of riboflavin/UVA combination (365 nm) in vitro for bacterial and fungal isolates: a potential new treatment for infectious keratitis. *Invest Ophthalmol Vis Sci*. 2008;49:3402–3408.
45. Khan YA, Kashiwabuchi RT, Martins SA, et al. Riboflavin and ultraviolet light A therapy as an adjuvant treatment for medically refractive Acanthamoeba keratitis: report of 3 cases. *Ophthalmology*. 2011;118:324–331.
46. Del Buey MA, Cristobal JA, Casas P, et al. Evaluation of in vitro efficacy of combined riboflavin and ultraviolet A for Acanthamoeba isolates. *Am J Ophthalmol*. 2012;153:399–404.

Effects of Multipurpose Contact Lens Care Solutions on the Adhesion of *Acanthamoeba* to Silicone Hydrogel Contact Lenses

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Purpose: To evaluate the effect of 7 multipurpose contact lens care solutions (MPSs) on the adhesion of *Acanthamoeba* (AC) to 5 silicone hydrogel contact lenses (SHCLs).

Methods: *Acanthamoeba castellanii* (ATCC50370) trophozoites were inoculated onto disks trimmed from SHCLs, Asmofilcon A, Galyfilcon A, Senofilcon A, Lotrafilcon B, and Balafilcon A. After 4-hour incubation, the number of adherent AC trophozoites on SHCL was counted under phase contrast microscopy. AC trophozoites mixed with 7 MPSs were inoculated onto Balafilcon A and incubated for 24 hours followed by direct counting, phase contrast microscopy, and scanning electron microscopy. AC cysts were also inoculated onto Balafilcon A followed by counting using phase contrast microscopy.

Results: Adhesion of AC trophozoites to Lotrafilcon B and Balafilcon A was 10 times higher in comparison with the other 3 SHCLs. Twenty four–hour treatment of AC trophozoites with Epica Cold, Epica Cold Aquamore, ReNu MultiPlus, OptiFree Plus, and Complete DoubleMoist reduced the numbers of adherent AC to less than 25% of control, whereas the numbers of AC treated with Complete AminoMoist and C3 SoftOne Moist was about 50% and 75% of control, respectively. Normal AC trophozoites without any treatments showed 25 times higher adhesion rates compared with normal AC cysts.

Conclusions: The adhesion rates of AC trophozoites to SHCL varied depending on the type of MPSs used. Appropriate uses of MPS could reduce adhesion rates of AC to SHCL and potentially decrease clinical rates of *Acanthamoeba* keratitis.

Key Words: Acanthamoeba, contact lens, multipurpose contact lens care solution

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Acanthamoeba spp are free-living protozoa that have a virtually ubiquitous distribution in water, soil, air, ventilation systems, and sewage systems.[1] *Acanthamoeba* (AC) can be an opportunistic pathogen of humans causing a potentially blinding corneal infection called *Acanthamoeba* keratitis (AK).[2] Because of difficulty in diagnosing and prolonged therapy, AK is thought to be the most recalcitrant among ocular infectious diseases.[3] It has also been established that AK is highly associated with soft contact lens (SCL) wear, and AC infection occurs through poor lens hygiene practices with noncompliant behaviors.[4,5] AC has 2 life cycle stages, the motile feeding trophozoite stage and the resistant dormant cyst stage.[1] Occasionally, AC cysts in dust, tap water, and soil contaminate contact lens storage cases and proliferate by feeding on gram-negative bacteria.[6] The SCL acts as a mechanical vector by transmitting AC onto the corneal surface, where AK is caused by invasion of AC.

Silicone hydrogel contact lenses (SHCLs) with higher oxygen transmissibility were introduced to the world contact lens market nearly a decade ago. Studies have found no significant difference in the incidence of microbial keratitis associated with SHCL and conventional SCL wear.[7,8] However, recent studies have shown that AC has a higher affinity to SHCLs, Lotrafilcon A and Balafilcon A in comparison with conventional SCL, Etafilcon A.[9] This high affinity of AC to SHCLs might be related to the high incidence of AK observed among SHCLs wearers.

However, most contact lens wearers use multipurpose contact lens care solutions (MPSs) for cleaning, rewetting, and disinfecting their conventional SCLs and SHCLs.[10] Several studies[11–16] conducted on the disinfecting efficacy of MPS against AC showed that most MPSs had limited efficacies against AC trophozoites and cysts. Moreover, Kilvington et al[15] showed that a specific MPS produced encystment of AC. However, to date, no studies have been published on the effects of MPSs on AC adhesion to contact lenses. Therefore, this study evaluated the effects of 7 commercially available MPSs on the adhesion of AC to an SHCL, Balafilcon A, having the highest affinity among the test SHCLs.

MATERIALS AND METHODS

Contact Lenses

Five SHCLs were purchased from commercial sources and used for the adhesion assay. The properties of lenses used

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in this study are shown in Table 1. After trimming at a 5 mm diameter using a trephine, test SHCL disks were placed at the bottom of 96-well plate (Falcon; BD Biosciences, Franklin Lakes, NJ) with the convex side up. For assessing the effects of MPSs, Balafilcon A was used as a test lens for the adhesion assay because of the high affinity of AC trophozoites to these lenses.

Multipurpose Solutions

Seven MPSs were purchased from commercial sources and were used within their expiration date. The compositions of these MPSs are shown in Table 2.

Preparations of AC Trophozoites and Cysts

Acanthamoeba castellanii strain (ATCC50370) was used for this study.[11–15] AC trophozoites and cysts were prepared as described previously.[17] Briefly, AC trophozoites were axenically cultured in a culture flask (Falcon; BD Biosciences) with peptone–yeast extract/glucose (PYG) medium (20.02 g of Bacto Proteose Peptone and 1.00 g of yeast extract in 950 mL of pure water, 50.0 mL of 2 M D(+)glucose, 10.0 mL of 0.4 M $MgSO_4 \cdot 7H_2O$, 8.0 mL of 0.05 M $CaCl_2$, 34.0 mL of 0.1 M sodium citrate $2H_2O$, 10.0 mL of 0.005 M $(Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 10.0 mL of 0.25 M $NaHPO_4 \cdot 7H_2O$, 10.0 mL of 0.25 M KH_2PO_4) at 32°C. The trophozoite suspension was prepared by gently scraping the culture flask with a cell scraper. The trophozoites were washed with one-quarter (1/4) strength Ringer solution (2.15 g of NaCl, 0.075 g of KCl, 0.076 g of $CaCl_2 \cdot 2H_2O$, and 1000 mL of pure water) twice by centrifugation at 300*g* (EX-125; TOMY, Tokyo, Japan) and resuspended in fresh 1/4 strength Ringer solution. The AC cysts were prepared by incubation in encystment medium (14.61 g of NaCl, 0.651 g of $MgCl_2 \cdot 6H_2O$, 0.053 g of $CaCl_2 \cdot 2H_2O$, and 1000 mL of pure water) for 14 days at 32°C.

Adhesion Assay of AC Trophozoites and Cysts to SHCL

The AC trophozoites were suspended in 1/4 strength Ringer solution, and the number of trophozoites was counted using a hemocytometer (Fuchs-Rosenthal; SLGC, Tokyo, Japan) under phase contrast microscope (IX70; Olympus Optical, Tokyo, Japan) and diluted to 1×10^5 cells per mL with 1/4 strength Ringer solution. Test disks were placed into a 96-well plate with 100 μL of 1/4 strength Ringer solution. Trophozoite suspension of 50 μL was inoculated into each well and the plate was stored at room temperature for 4 hours. After washing each well twice with 2 mL of saline, the total number of AC adhered to test disks was counted using the phase contrast microscope at ×40 magnification. Seven lenses of each type were used in this experiment. The AC cysts were also tested.

Adhesion Assay of MPS-Treated AC to SHCL

For the MPS study, trimmed Balafilcon A was used as the test disk. AC trophozoite suspension of 50 μL (1×10^5 cells per mL in 1/4 strength Ringer solution) was inoculated into a 96-well plate placed with a test disk. After adding 100 μL of MPS solution to be tested, the plate was stored at room temperature for 24 hours. The total number of AC adhered to test disks was counted as described in the previous section. Seven lenses were used for each MPS solution.

Phase Contrast and Scanning Electron Microscopes of Adherent AC

AC trophozoite suspension of 50 μL (5×10^5 cells per mL in 1/4 strength Ringer solution) was seeded on a test disk placed into a 96-well plate. After adding 100 μL of test solutions, the plate was stored at room temperature for 24 hours. After washing the disk twice with 1/4 strength Ringer solution, the adherent AC was photographed with a digital camera equipped to the phase contrast microscope. The test disk was subsequently fixed with 2.5% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA) in 100 mM sodium cacodylate buffer (pH 7.4) for 1 hour at 4°C, and then postfixed with 1% OsO_4 in the same buffer for 1 hour at 4°C. After standard graded dehydration with ethanol (50%, 70%, 80%, 85%, 90%, 95%, and 100%), the specimens were dried by the critical point method (HCP-1; Hitachi, Tokyo, Japan). Dried specimens were mounted on stubs with conductive adhesive tape and conventionally coated with a thin layer of palladium–platinum in a sputter coater (emscope SC500; Meiwa, Osaka, Japan). The specimens were viewed with a scanning electron microscope (SEM; S-4800; Hitachi, Tokyo, Japan) at 5 kV.

TABLE 1. Silicone Hydrogel Contact Lenses Tested

USAN	SHCL	Manufacturer	Water Content (%)	Surface Treatment or Applied Technology
Asmofilcon A	PremiO	Menicon	40	Nanogloss
Galyfilcon A	Acuvue Advance	Johnson & Johnson	47	Hydra clear (PVP)
Senofilcon A	Acuvue Oasys	Johnson & Johnson	38	None
Lotrafilcon B	O2 Optics	CIBA Vision	33	Plasma coating
Balafilcon A	Pure Vision	Bausch & Lomb	36	Plasma oxidation

USAN, United States adopted names; PVP, polyvinylpyrrolidone.

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TABLE 2. Multipurpose Solutions Tested

MPS	Manufacturer	Preservatives	Surfactants	Isotonic Agents	Boric Acid
MeniCare Soft	Menicon	PHMB (1 ppm)	Macrogolglycerol hydroxystearate	Glycine	−
Epica Cold Aquamore	Menicon	PHMB (1 ppm)	Macrogolglycerol hydroxystearate	Glycine	−
ReNu MultiPlus	Bausch & Lomb	PHMB (1.1 ppm)	Poloxamine	NaCl	+
OptiFree Plus	Alcon	Polyquad (11 ppm)	Poloxamine	NaCl	+
Complete AminoMoist	AMO	PHMB (1 ppm)	Poloxamer	NaCl	−
Complete DoubleMoist	AMO	PHMB (1 ppm)	Poloxamer	NaCl	−
C3 SoftOne Moist (g)	Rohto	PHMB (1 ppm)	Poloxamer	NaCl	−

PHMB, polyhexamethylene biguanide.

Statistical Analysis

Statistical analysis of the number of adherent AC trophozoites and cysts to SHCLs was performed using analysis of variance (ANOVA) with the Scheffe test or nonpaired *t* test via Excel Stat (Microsoft, Redmond, WA).

RESULTS

Adhesion of AC Trophozoites and Cysts to SHCLs

Figure 1 shows the number of AC trophozoites adherent to 5 test SHCLs. The numbers of adherent AC trophozoites to Lotrafilcon B and Balafilcon A were 212 ± 119 and 232 ± 89 cells per disk, respectively. These numbers were significantly higher (ANOVA with Scheffe, $P < 0.01$) in comparison with the numbers of adherent AC trophozoites to Asmofilcon A, Galyfilcon A, and Senofilcon A, which were 22 ± 6, 38 ± 19, and 16 ± 14 cells per disk, respectively.

Balafilcon A was used in the next adhesion assay because of its high adherence to AC trophozoites. The number of AC trophozoites adhered to Balafilcon A was 175 ± 87, which was 25 times higher than that of AC cysts, 7 ± 4 (nonpaired *t* test, $P < 0.0001$).

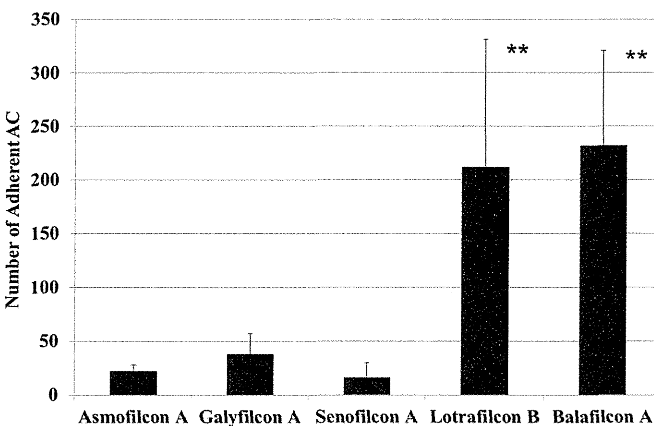

FIGURE 1. Number of adherent *Acanthamoeba castellanii* trophozoites to SHCL. AC trophozoites were inoculated onto silicone hydrogel contact lenses and incubated for 4 hours. **Significant differences ($P < 0.01$) between Asmofilcon A.

Adhesion of AC Treated With MPS to SHCL

Figure 2 shows the adhesion number of AC to Balafilcon A with MPS treatment. The numbers of adherent AC treated with MeniCare Soft, Epica Cold Aquamore, ReNu MultiPlus OptiFree Plus, and Complete DoubleMoist were 10 ± 6, 28 ± 17, 9 ± 4, 34 ± 16, and 32 ± 19 cells per disk, respectively. These numbers were significantly (ANOVA with Scheffe, $P < 0.01$) lower compared with the 1/4 strength Ringer solution–treated disk (control), 143 ± 60 cells per disk. Complete AminoMoist–treated AC showed relatively high adherence (64 ± 39 cells per disk), yet still significantly lower than the control (ANOVA with Scheffe, $P < 0.05$). The highest number of AC adherence was observed with C3 SoftOne Moist (88 ± 59 cells per disk).

Morphology of AC Treated With MPS

Figure 3 shows phase contrast microscopic images of adherent AC to Balafilcon A treated with MPS. The number of adherent AC was higher in the control (1/4 strength Ringer solution treatment) compared with AC treated with Epica Cold, Aquamore, or ReNu MultiPlus (Figs. 3A–D). One-quarter strength Ringer solution–treated trophozoites kept their normal shape (Fig. 3A) compared with MPS-treated trophozoites, which become spherical (precyst) after 24 hours. Both trophozoite and precyst were observed at the same time in AC treated with OptiFree Plus, Complete AminoMoist, Complete DoubleMoist, or C3 SoftOne Moist (Figs. 3E–H).

Figure 4 shows representative SEM images of adherent AC to Balafilcon A treated with MPS. AC treated with 1/4 strength Ringer solution, OptiFree Plus, Complete DoubleMoist, or C3 SoftOne Moist (Figs. 4A–H) kept their trophozoite shape with the expanding filopodia onto the lens surface. Epica Cold-, Aquamore-, or ReNu MultiPlus–treated AC (Figs. 4B–D) changed into cystic shapes (precyst form). Complete AminoMoist–treated AC (Fig. 4F) showed intermediate shape between trophozoite and cyst or deformed precyst form.

DISCUSSION

Bacterial adhesion to conventional SCL and SHCL has been studied extensively using *Pseudomonas aeruginosa*,

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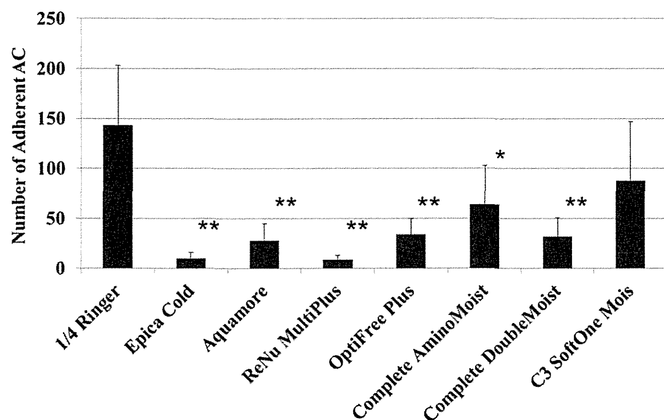

FIGURE 2. Number of adherent *Acanthamoeba castellanii* treated with MPS to Balafilcon A. AC trophozoites were mixed with 7 MPSs and inoculated onto Balafilcon A and incubated for 24 hours. *Significant differences ($P < 0.05$) between 1/4 strength Ringer solution; **significant differences ($P < 0.01$) between 1/4 strength Ringer solution.

Serratia marcescens, *Staphylococcus aureus*, and *Staphylococcus epidermidis* (SE), commonly detected in contact lens cases.[18,19] Santos et al[20] compared adhesiveness of SE to unworn and worn Etafilcon A (Acuvue), Galyfilcon A (Acuvue Advance), Balafilcon A (Pure Vision), Lotrafilcon A (Focus Night & Day), and Lotrafilcon B (O2Optics) and showed reduced microbial adhesions to worn SHCLs compared with unworn lenses. However, the number of SE detected on worn conventional SCL, Etafilcon A, was significantly higher in comparison with unworn lenses. Furthermore, Santos et al[20] demonstrated that the difference in SE number is due to lipid adsorption and microbial adhesions, which change conventional SCL and SHCL surface hydrophobicities.

Beattie et al[9,21] conducted studies on AC adherence to conventional SCL and SHCL in vitro, biofilm coating, and lens wear. Their results showed that a significantly higher number of AC adhered to SHCL, Lotrafilcon A and Balafilcon A compared with conventional SCL, Etafilcon A. However, there were no differences in AC number between unworn and worn Lotrafilcon A. In addition, no *Pseudomonas aeruginosa* biofilm coating was found. Our results showed a direct correlation between contact lens brands and adhesion of AC trophozoites. On all SHCLs tested, adhesion of AC trophozoites was high with Lotrafilcon B and Balafilcon A. This result is consistent with reports that showed that the attachment of AC to contact lenses is influenced by various parameters, including lens material properties, ionicity, water contents, surface hydrophobicity, and protein-adsorption and lipid-adsorption properties.[22] Recently, Beattie and Tomlinson[23] have reported that treatment of Lotrafilcon A surface with plasma reduces its hydrophobicity while significantly enhancing AC adhesion. They also suggested the possibility that the increased attachment found with Balafilcon A might be an inherent characteristic of the polymer or a side effect of the surface treatment procedure to the lens;

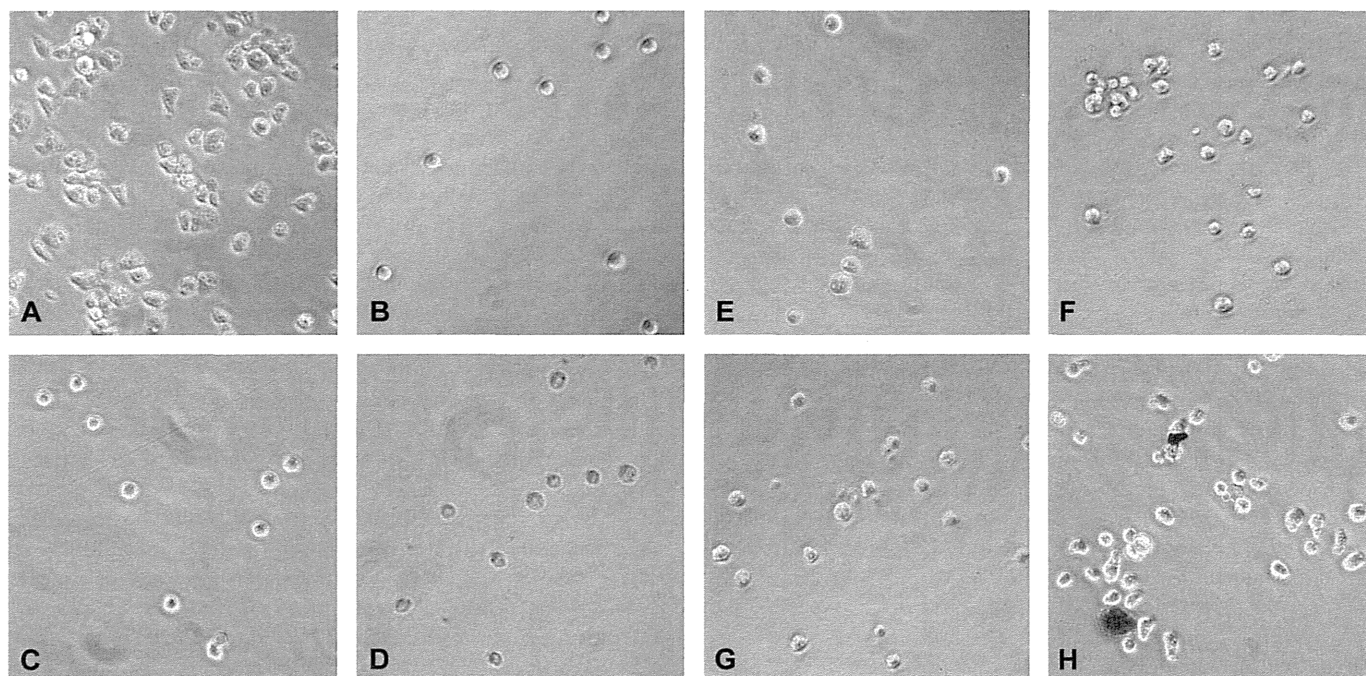

FIGURE 3. Phase contrast microscopic images of adherent *Acanthamoeba castellanii* treated with MPS to Balafilcon A. A, One-quarter Ringer solution treatment for 24 hours. B, Epica Cold treatment for 24 hours. C, Aquamore treatment for 24 hours. D, ReNu MultiPlus treatment for 24 hours. E, OptiFree Plus treatment for 24 hours. F, Complete AminoMoist treatment for 24 hours. G, Complete DoubleMoist treatment for 24 hours. H, C3 SoftOne Moist treatment for 24 hours (original magnification ×200). AC trophozoites were mixed with 7 MPSs and inoculated onto Balafilcon A and incubated for 24 hours.

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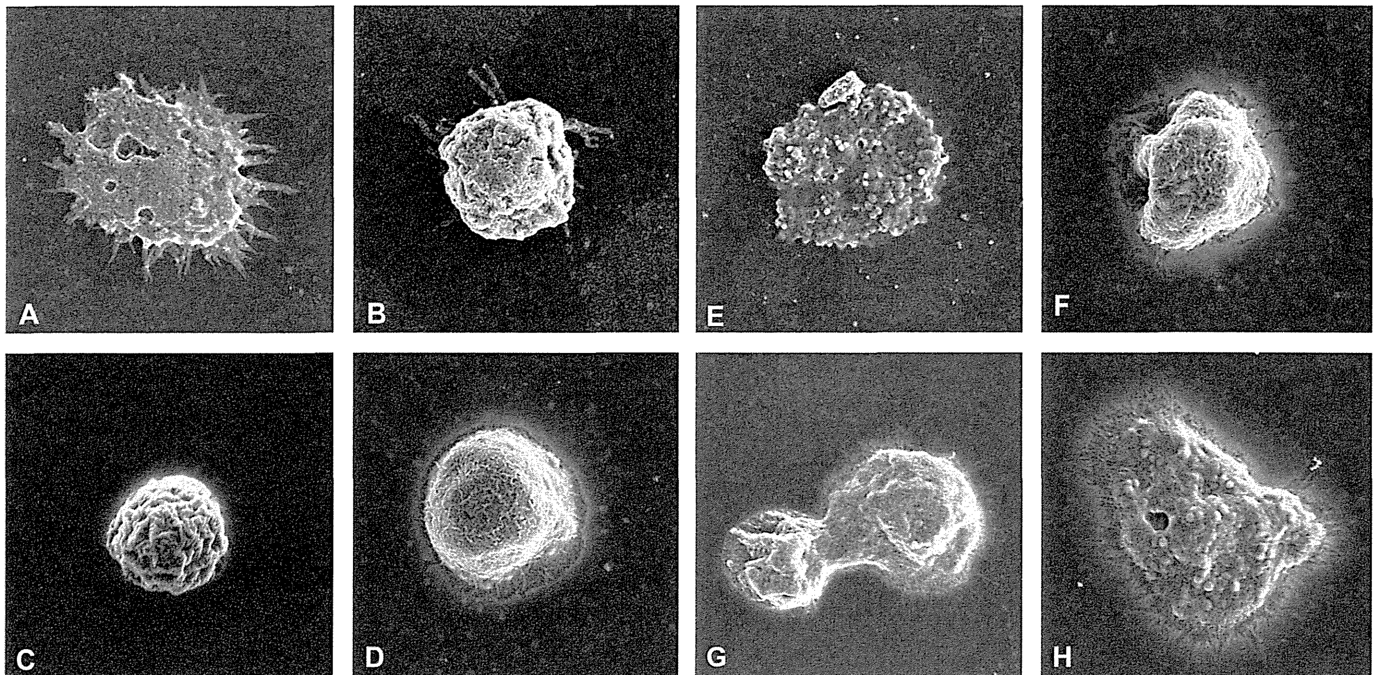

FIGURE 4. SEM images of adherent *Acanthamoeba castellanii* treated with MPS to Balafilcon A. A, One-quarter Ringer solution treatment for 24 hours (original magnification ×2500). B, Epica Cold treatment for 24 hours (original magnification, ×5000). C, Aquamore treatment for 24 hours (original magnification, ×3000). D, ReNu MultiPlus treatment for 24 hours (original magnification ×4000). E, OptiFree Plus treatment for 24 hours (original magnification ×3500). F, Complete AminoMoist treatment for 24 hours (original magnification ×5000). G, Complete DoubleMoist treatment for 24 hours (original magnification ×2500). H, C3 SoftOne Moist treatment for 24 hours (original magnification, ×3000). AC trophozoites were mixed with 7 MPSs and inoculated onto Balafilcon A and incubated for 24 hours.

however, it is very difficult to specify which material properties of SHCLs may affect the AC adhesion behavior. Additional studies may be needed to clarify the high affinity of AC to Lotrafilcon B and Balafilcon A.

Kilvington[24] has compared the adherence of AC trophozoites and cysts with 4 types of SCLs. Food and Drug Administration group I, ordinal poly-2-hydroxyethyl methacrylate lenses showed the highest adhesion for both trophozoites and cysts. The results of Kilvington's studies showed that adhesion of trophozoites was 5 to 10 times higher than adhesion of cysts. These results are similar to our results, which showed that adhesion of trophozoites is 25 times higher than adhesion of cysts. It is possible that adherence of trophozoites to the contact lens is mediated by long slender pseudopods, termed filopodia, which are absent in the cyst forms.[25]

Usually AC cysts are experimentally prepared by incubation with encystment medium for more than 1 week[25]; however, we previously reported morphological changes of AC after 4-hour treatment with 2 MPSs, MeniCare Soft (Epica Cold) and ReNu MultiPlus.[17] SEM analysis of AC morphology revealed that MeniCare Soft– and ReNu MultiPlus–treated trophozoite changed into deformed cyst shapes, whereas Complete DoubleMoist– and C3 SoftOne Moist–treated trophozoites kept their normal shape.[17] Four-hour exposure to Complete DoubleMoist and C3 SoftOne Moist had no effects on AC adhesion compared with the control, 1/4 strength Ringer solution.[17] These results are similar to our present data that showed deformed AC trophozoites after 24 hours when treated with Epica Cold, Aquamore, and ReNu MultiPlus. AC trophozoite treated with OptiFree Plus, Complete DoubleMoist, and C3 SoftOne Moist kept their forms. Recently, Lonnen et al[12] have compared disinfection efficacy of Epica Cold, Aquamore, OptiFree Plus, and C3 SoftOne Moist using *A castellanii* (ATCC50370) and demonstrated that Epica Cold and Aquamore have significantly higher disinfecting efficacies against AC than OptiFree Plus and C3 SoftOne Moist. Using number enumeration technique, Beattie et al[14] have also reported that ReNu MultiPlus had the highest efficacy compared with OptiFree Express, and Complete DoubleMoist had the lowest efficacy on AC trophozoites. These results showed that high-efficacy MPSs could induce the deformation of AC trophozoites and reduced AC adhesion to contact lenses.

In conclusion, the results from this study showed that AC adhesion rates varied depending on the type of MPS used. These results also indicated that appropriate uses of MPS could reduce the adhesion rates of AC to SHCL and, therefore, decrease AK associated with contact lens wearing.

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REFERENCES

1. Ma P, Visvesvara GS, Martinez AJ, et al. Naegleria and Acanthamoeba infections: review. *Rev Infect Dis*. 1990;12:490–513.
2. Clarke DW, Niederkorn JY. The pathophysiology of Acanthamoeba keratitis. *Trends Parasitol*. 2006;22:175–180.
3. McCulley JP, Alizadeh H, Niederkorn JY. The diagnosis and management of Acanthamoeba keratitis. *CLAO J*. 2000;26:47–51.
4. Radford CF, Minassian DC, Dart JK. Disposable contact lens use as a risk factor for microbial keratitis. *Br J Ophthalmol*. 1998;82:1272–1275.
5. Joslin CE, Tu EY, Shoff ME, et al. The association of contact lens solution use and Acanthamoeba keratitis. *Am J Ophthalmol*. 2007;144:169–180.
6. Seal DV, Kirkness CM, Bennett HG, et al. Acanthamoeba keratitis in Scotland: risk factors for contact lens wearers. *Cont Lens Anterior Eye*. 1999;22:58–68.
7. Schornack MM, Faia LJ, Griepentrog GJ. Pseudomonas keratitis associated with daily wear of silicone hydrogel contact lenses. *Eye Contact Lens*. 2008;34:124–128.
8. Lim L, Loughnan MS, Sullivan LJ. Microbial keratitis associated with extended wear of silicone hydrogel contact lenses. *Br J Ophthalmol*. 2002;86:355–357.
9. Beattie TK, Tomlinson A, McFadyen AK. Attachment of Acanthamoeba to first- and second-generation silicone hydrogel contact lenses. *Ophthalmology*. 2006;113:117–125.
10. Morgan PB, Woods CA, Knajian R, et al. International contact lens prescribing in 2008. *Contact Lens Spectrum*. 2009;36:28–32.
11. Borazjani RN, Kilvington S. Efficacy of multipurpose solutions against Acanthamoeba species. *Cont Lens Anterior Eye*. 2005;28:169–175.
12. Lonnen J, Heaselgrave W, Nomachi M, et al. Disinfection efficacy and encystment rate of soft contact lens multipurpose solutions against Acanthamoeba. *Eye Contact Lens*. 2010;36:26–32.
13. Shoff M, Rogerson A, Schatz S, et al. Variable responses of Acanthamoeba strains to three multipurpose lens cleaning solutions. *Optom Vis Sci*. 2007;84:202–207.
14. Beattie TK, Seal DV, Tomlinson A, et al. Determination of amoebicidal activities of multipurpose contact lens solutions by using a most probable number enumeration technique. *J Clin Microbiol*. 2003;41:2992–3000.
15. Kilvington S, Heaselgrave W, Lally JM, et al. Encystment of Acanthamoeba during incubation in multipurpose contact lens disinfectant solutions and experimental formulations. *Eye Contact Lens*. 2008;34:133–139.
16. Kobayashi T, Gibbon L, Mito T, et al. Efficacy of commercial soft contact lens disinfectant solutions against Acanthamoeba. *Jpn J Ophthalmol*. 2011;55:547–557.
17. Imayasu M, Uno T, Ohashi Y, et al. Effects of multipurpose contact lens care solutions on the adhesiveness of Acanthamoeba to corneal epithelial cells. *Eye Contact Lens*. 2009;35:246–250.
18. Borazjani RN, Levy B, Ahearn DG. Relative primary adhesion of Pseudomonas aeruginosa, Serratia marcescens and Staphylococcus aureus to HEMA-type contact lenses and an extended wear silicone hydrogel contact lens of high oxygen permeability. *Cont Lens Anterior Eye*. 2004;27:3–8.
19. Henriques M, Sousa C, Lira M, et al. Adhesion of Pseudomonas aeruginosa and Staphylococcus epidermidis to silicone-hydrogel contact lenses. *Optom Vis Sci*. 2005;82:446–450.
20. Santos L, Rodrigues D, Lira M, et al. Bacterial adhesion to worn silicone hydrogel contact lenses. *Optom Vis Sci*. 2008;85:520–525.
21. Beattie TK, Tomlinson A, McFadyen AK, et al. Enhanced attachment of acanthamoeba to extended-wear silicone hydrogel contact lenses: a new risk factor for infection? *Ophthalmology*. 2003;110:765–771.
22. Iwata M, Ohno S, Kawai T, et al. In vitro evaluation of lipids adsorbed on silicone hydrogel contact lenses using a new gas chromatography/-mass spectrometry analytical method. *Eye Contact Lens*. 2008;34:272–280.
23. Beattie TK, Tomlinson A. The effect of surface treatment of silicone hydrogel contact lenses on the attachment of Acanthamoeba castellanii trophozoites. *Eye Contact Lens*. 2009;35:316–319.
24. Kilvington S. Acanthamoeba trophozoite and cyst adherence to four types of soft contact lens and removal by cleaning agents. *Eye (Lond)*. 1993;7:535–538.
25. Kilvington S, Anger C. A comparison of cyst age and assay method of the efficacy of contact lens disinfectants against Acanthamoeba. *Br J Ophthalmol*. 2001;85:336–340.

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Use of 5-Cyano-2,3-Ditolyl-Tetrazolium Chloride Staining as an Indicator of Biocidal Activity in a Rapid Assay for Anti-*Acanthamoeba* Agents

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The usefulness of 5-cyano-2,3-ditolyl-tetrazolium chloride (CTC) staining to determine the respiratory activity of *Acanthamoeba* was evaluated in this study. *Acanthamoeba* trophozoites and cysts have a red fluorescence after staining with CTC. To determine the effectiveness of CTC staining as a CTC biocidal assay for *Acanthamoeba*, the trophozoites and cysts of *Acanthamoeba castellanii* (ATCC 5037) were treated with serial concentrations of disinfectant solutions, namely, polyhexamethylene biguanide (PHMB) and commercial soft contact lens (SCL) disinfectant solutions. The treated *Acanthamoeba* organisms were stained with CTC, and their respiratory activity was determined by the intensity of fluorescence in a fluorescence microplate reader. The survival rates of the same samples were determined by a culture-dependent biocidal assay using the Spearman-Karber method. Our results showed that the respiratory activities determined by the CTC biocidal assay and the survival rates determined by the culture-dependent biocidal assay for *Acanthamoeba* trophozoites and cysts decreased in a dose-dependent way after PHMB treatments, and the results were significantly correlated ($r = 0.83$ and $P < 0.01$ for trophozoites; $r = 0.60$ and $P < 0.01$ for cysts; Spearman rank correlation test). The respiratory activities in the trophozoites and cysts treated with SCL disinfectant solutions were significantly correlated with the survival rate ($r = 0.70$ and $P < 0.01$ for trophozoites; $r = 0.64$ and $P < 0.01$ for cysts; Spearman rank correlation test). The significant correlation of the results indicated that the CTC biocidal assay can be used as an alternative method to a culture-dependent biocidal assay. The CTC biocidal assay is a rapid and simple method to test the effectiveness of disinfectant solutions against *Acanthamoeba* trophozoites and cysts.

Acanthamoeba keratitis (AK) is painful and potentially blinding (22). In recent years, AK has been associated with contact lens-related corneal diseases (29). The recent increase in the incidence of AK has been attributed to several factors, including the rising number of soft contact lens (SCL) wearers and the widespread noncompliance with the cleaning and rinsing regimens for SCLs (9, 13, 17, 19). In addition, the use of SCL disinfectant solutions that are not effective is also suspected to be linked to the increase in cases of AK (6).

The situation that SCL disinfectants may not be effective against *Acanthamoeba* has arisen partially because there is no standardized method to evaluate the effectiveness of lens care disinfectants against *Acanthamoeba*. The International Organization for Standardization (ISO) has adopted the Stand Alone test (ISO 14729), a standard method for testing the disinfectant efficacy of lens care products. However, this method does not include a protocol specifically for *Acanthamoeba*. Thus, a standard method for testing the disinfecting efficacy of lens care products against *Acanthamoeba* is needed to determine the effectiveness of new disinfectant lens care products against *Acanthamoeba*.

Traditionally, culture-dependent methods have been used to evaluate the effectiveness of various disinfectants against *Acanthamoeba* (1, 2, 11, 12, 15, 20, 24). Among these, culture-dependent biocidal assays using the most-probable-number (MPN) method or the Spearman-Karber method have been considered suitable methods to quantify the number of living organisms (1, 18, 24). Although conventional culture-dependent methods have been shown to be reliable for detecting surviving organisms after exposure to disinfectants, the requirement of long-term cultivation may be limiting for the development of new disinfectants (1, 11, 18, 24). In fact, the previously reported culture-dependent biocidal assay requires 1 week for trophozoites and 3 weeks for cysts to be detected (18). Therefore, a rapid method to test the efficacy of disinfectant solutions would be useful for laboratory investigations, especially for testing the efficacy of new disinfectants.

5-Cyano-2,3-ditolyl-tetrazolium chloride (CTC) is a redox dye that is widely used to determine the respiratory activity of bacteria (27, 30). CTC is a soluble crystal that forms a nearly colorless nonfluorescent solution. In the electron transport system, tetrazolium salts function as artificial redox partners instead of the final electron acceptor, oxygen (7). Respiring bacteria placed in CTC solution will take up the CTC and reduce it to insoluble formazan (CTC formazan), which accumulates in the cells. On the other hand, dead or inactive cells show no accumulation of CTC formazan (26, 27). Because the dye competes with the terminal electron acceptor, it will eventually poison the cells once the reduction processes are completed. Therefore, CTC staining represents an index of the respiratory activity of the cell at the time of observation (14). CTC formazan emits a red fluorescence (emission peak, 630 nm) when excited by a blue light (peak, 480 nm). Thus, it is

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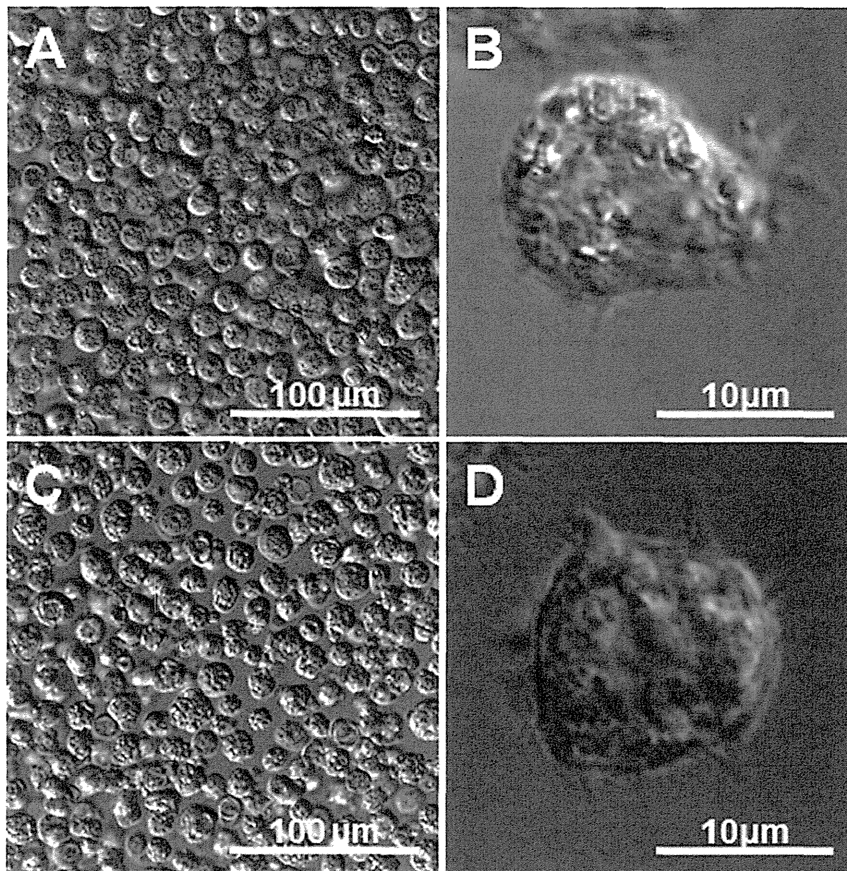

FIG 1 *Acanthamoeba castellanii* trophozoites stained with CTC. Merged fluorescence images of red-fluorescing CTC formazan and differential interference contrast images of trophozoites stained with CTC are shown. (A) CTC formazan accumulates within trophozoites, as shown by red fluorescence. (B) Trophozoite from panel A at higher magnification. (C) Inhibition of respiration of trophozoites by addition of sodium azide. (D) Respiration-inhibited trophozoite from panel C at higher magnification.

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possible to distinguish fluorescence-labeled respiring active cells from inactive cells by fluorescence microscopy. It has been reported that the bacterial respiratory activity assessed by CTC staining is well correlated with bacterial viability units such as CFU (7, 25).

However, there have been few reports on the application of CTC staining for protozoans (14), and it has not been used for *Acanthamoeba* spp. Thus, the purpose of this study was to determine whether CTC staining can be used for rapid biocidal assay of *Acanthamoeba*. To accomplish this, we first investigated whether it is possible to determine the respiratory activity of *Acanthamoeba* by CTC staining. A biocidal assay for *Acanthamoeba* with CTC staining was then performed, and the respiratory activities obtained were compared to the survival rates determined by a culture method using the Spearman-Karber method.

MATERIALS AND METHODS

***Acanthamoeba* trophozoites and cysts.** We used *Acanthamoeba castellanii* (ATCC 50370) for this study. Trophozoites were cultured in peptone-yeast extract-glucose (PYG) medium (ATCC medium 712) in tissue culture flasks (Becton Dickinson, Tokyo, Japan) at 25°C. Encystment was induced by transferring the trophozoites from PYG medium to Neff's constant-pH encystment medium (23) and incubating the trophozoites for at least 2 weeks at 25°C. All procedures involving the organisms were carried out in biosafety level 2 laboratories.

CTC staining. *Acanthamoeba* trophozoites were collected from the solutions in the flasks by centrifugation. Centrifugation was carried out for 10 min at 150 × *g* throughout the experiments for both trophozoites and cysts. The trophozoites were counted with a hemocytometer under a phase-contrast microscope, and they were suspended in phosphate-buffered saline (PBS) at 2×10^6 trophozoites in 1.8 ml of PBS. The amoeba suspension was divided into two portions (900 µl each), and 100 µl of H_2O was added to one portion and 100 µl of sodium azide solution (20 mg/ml) was added to the other portion. The organisms that had their respiration inhibited by sodium azide were used as negative controls (14). CTC staining was performed on each portion by use of a Bacstain-CTC rapid staining kit (Dojindo Laboratories, Kumamoto, Japan) for 30 min at 25°C according to the manufacturer's instructions. After staining, the samples were fixed by adding 1 ml of paraformaldehyde (4% in PBS) for 30 min at 4°C.

Acanthamoeba cysts were collected from the solutions in the flasks by centrifugation and then counted with a hemocytometer under a phase-contrast microscope. They were then suspended in 10 ml of PYG medium (2×10^6 cysts in 10 ml of PYG medium) and preincubated for 16 h at 25°C to restore the respiratory activity of the organisms. After preincubation, the cysts were collected by centrifugation, stained by CTC with or without sodium azide, and fixed as described above.

The fixed trophozoites and cysts were collected by centrifugation and suspended in 1 ml of PBS. Two hundred microliters of the amoeba suspension was transferred to each well of 96-well glass-bottom plates (Asahi Techno Glass, Chiba, Japan) and examined by fluorescence

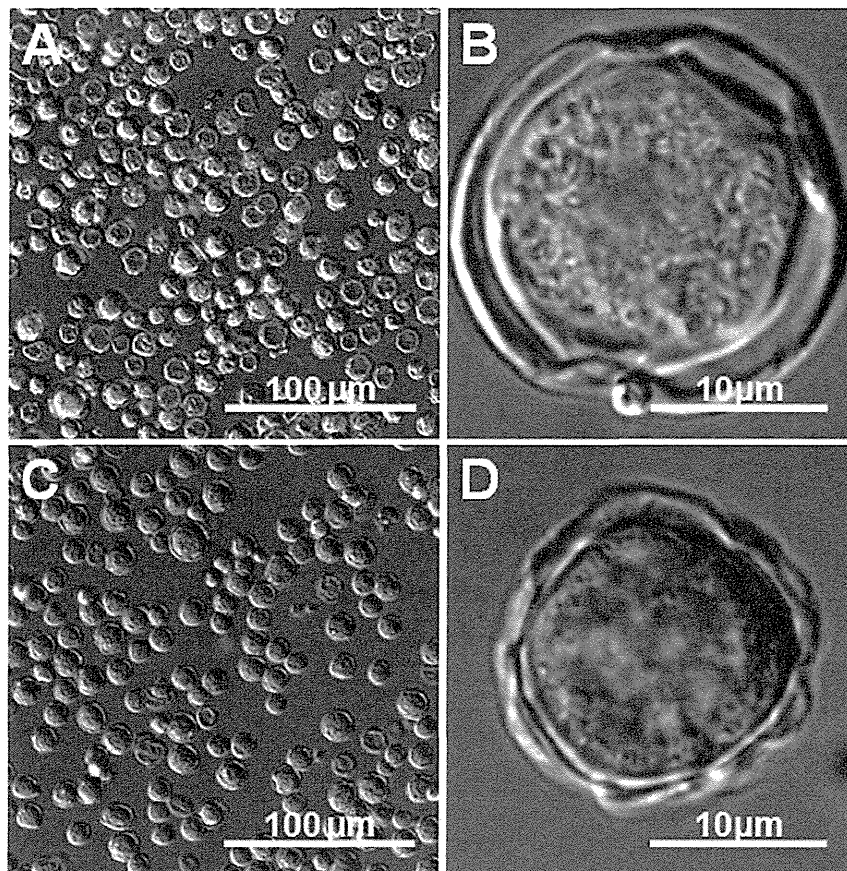

FIG 2 *Acanthamoeba castellanii* cysts stained with CTC. Merged fluorescence images of red-fluorescing CTC formazan and differential interference contrast images of cysts stained with CTC are shown. (A) CTC formazan accumulates within cysts, as shown by red fluorescence. (B) Cyst from panel A at higher magnification. (C) Inhibition of respiration of cysts by addition of sodium azide. (D) Respiration-inhibited cyst from panel C at higher magnification.

microscopy (excitation wavelength, 480 nm; emission wavelength, 630 nm).

Disinfectant treatments for *Acanthamoeba*. Polyhexamethylene biguanide (PHMB) was diluted with one-quarter-strength (1/4) Ringer's solution (Nihon Pharmaceutical Co., Ltd., Tokyo, Japan) to final concentrations of 0.1, 0.2, 0.5, 1, 2, 5, and 10 ppm for trophozoites and 1, 10, 100, and 300 ppm for cysts. Commercial SCL disinfectant solutions used were Complete Double Moist (AMO, Inc.) (MPS1), Optifree Plus (Alcon Japan, Ltd.) (MPS2), Renu Fresh (Bausch and Lomb Japan Company, Ltd.) (MPS3), Bioclen First Care, and CT (Ophtecs Corp.) (povidone iodine solution). MPS1 contained 1.0 ppm PHMB as the disinfectant. MPS3 contained 1.1 ppm PHMB, and MPS2 contained 11 ppm polydronium chloride (Polyquad). The povidone iodine solution was made by adding the attached disinfecting neutralizing tablet to the solution at the onset of disinfection according to the manufacturer's instructions.

The trophozoites or cysts were collected from the solutions in the flasks, and after centrifugation, the organisms were suspended in 1/4 Ringer's solution at a concentration of 5×10^6 organisms/ml. A 400-μl sample of the amoeba suspension was added to 40 ml of each disinfectant solution, to a final concentration of 5×10^4 organisms/ml. Control samples were also prepared in 1/4 Ringer's solution. Subsequently, each sample was incubated at 25°C for 4 h in a 50-ml conical tube (Becton Dickinson). After exposure to the disinfectant, the amoebal respiratory activity was determined by the CTC biocidal assay and the survival rate was determined by the culture-dependent biocidal assay using the Spearman-Karber method.

CTC biocidal assay. The *Acanthamoeba* trophozoites or cysts that were treated with the disinfectant solutions or 1/4 Ringer's solution (control) were collected by centrifugation and stained and fixed as described above. Organisms that were stained with CTC following fixation were collected by centrifugation and suspended in 1 ml of PBS. A 200-μl sample of the amoeba suspension was transferred to each well of a 96-well plate (Corning International Inc., Tokyo, Japan), and the fluorescence intensity was measured with a fluorescence microplate reader (FlexStation 3; Molecular Devices, Sunnyvale, CA) (excitation wavelength, 480 nm; emission wavelength, 630 nm). The samples that had sodium azide added to inhibit respiration were used as negative controls. To normalize the fluorescence intensity, the fluorescence intensity of the negative control was subtracted from the value of the test sample (14). Respiratory activity is presented as a percentage of the 1/4 Ringer's solution control level.

Culture-dependent biocidal assay using the Spearman-Karber method. The culture-dependent biocidal assay using the Spearman-Karber method was performed as described in detail previously (18). Briefly, after exposure to disinfectant or 1/4 Ringer's solution, 100 μl of the test solution was mixed with 900 μl of Dey-Engley neutralizing broth (Sigma, St. Louis, MO) and with 10-fold serial dilutions of each test solution in PYG medium. This resulted in four dilutions, with theoretical maximum final concentrations of 5×10^3, 5×10^2, 5×10^1, and 5×10^0 amoeba/ml. Four 200-μl samples of each dilution were transferred to separate wells in a 96-well plate (Corning International Inc., Tokyo, Japan) and incubated at 25°C. Samples containing trophozoites were incubated for 1 week, while those containing cysts were incubated for 3 weeks. At the end of the incubation period, amoebal growth in the wells was confirmed using a phase-contrast microscope. The number of surviving organisms was counted for each test solution by using the Spearman-Karber equation as

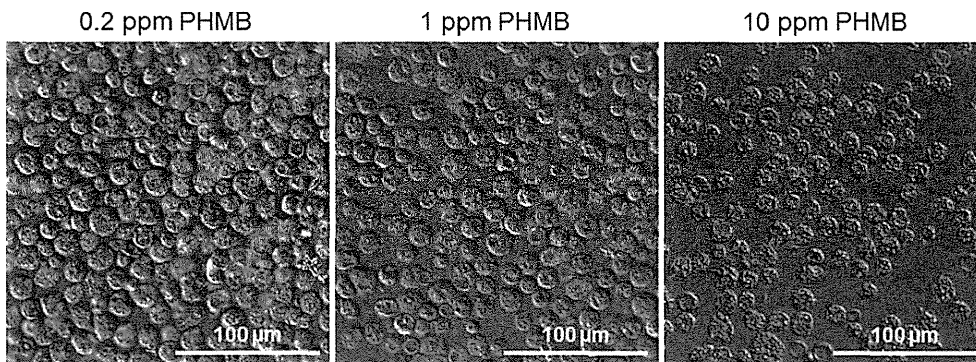

FIG 3 CTC staining of PHMB-treated *Acanthamoeba* trophozoites. Merged fluorescence images of red-fluorescing CTC formazan and differential interference contrast images of trophozoites stained with CTC after PHMB treatments are shown. CTC staining was performed on trophozoites treated with PHMB (0.2, 1, or 10 ppm) for 4 h at 25°C.

described previously (10). The survival rate is presented as a percentage of that in the 1/4 Ringer's solution control.

Statistical analysis. The Spearman rank correlation coefficient was used to determine the relationship between respiratory activity (CTC biocidal assay) and survival rate (culture-dependent biocidal assay).

RESULTS

CTC staining. Our results showed that CTC formazan, which has a red fluorescence when excited by blue light, accumulated inside *Acanthamoeba castellanii* trophozoites (Fig. 1A and B) but not in trophozoites exposed to sodium azide (Fig. 1C and D). The accumulation of CTC formazan was also observed in *Acanthamoeba* cysts after 16 h of preincubation in PYG medium (Fig. 2A and B) but not in cysts exposed to sodium azide (Fig. 2C and D). On the other hand, no CTC formazan accumulation was observed in *Acanthamoeba* cysts without preincubation in PYG medium (data not shown), indicating that the respiratory activity of the dormant cysts was restored by 16 h of preincubation in PYG medium.

CTC biocidal assay. The trophozoites exposed to 0.2 ppm of PHMB appeared red by fluorescence, indicating that most of the trophozoites were respiring (Fig. 3). On the other hand, fluorescence was not observed in the trophozoites exposed to 10 ppm of PHMB, and only a weak fluorescence was detected in the trophozoites exposed to 1 ppm of PHMB (Fig. 3). The respiratory activity determined by the CTC biocidal assay and the survival rate determined by the culture-dependent biocidal assay for trophozoites treated with PHMB are shown in Fig. 4. The respiratory activities were 87.5%, 61.3%, 18.0%, 9.1%, 1.2%, 2.0%, and 0% of the control level and the survival rates were 91.4%, 54.9%, 27.0%, 11.1%, 2.7%, 0.02%, and 0% of the control level for samples treated with 0.1, 0.2, 0.5, 1, 2, 5, and 10 ppm PHMB, respectively. Thus, the respiratory activity (CTC biocidal assay) and the survival rate (culture-dependent biocidal assay) were reduced after PHMB treatment, in a dose-dependent manner, and a significant positive correlation between the respiratory activity and the survival rate was found for trophozoites treated with PHMB ($r = 0.83$ and $P < 0.01$; Spearman rank correlation test).

The amoebal respiratory activities and survival rates after a 4-h exposure to SCL disinfectant solutions are shown in Fig. 5. The respiratory activities of the trophozoites were 48.0%, 30.4%, 0%, and 0% of the control level and the survival rates were 68.5%, 26.2%, 1.1%, and 0.04% of the control level for the samples treated with MPS1, MPS2, MPS3, and povidone iodine solution,

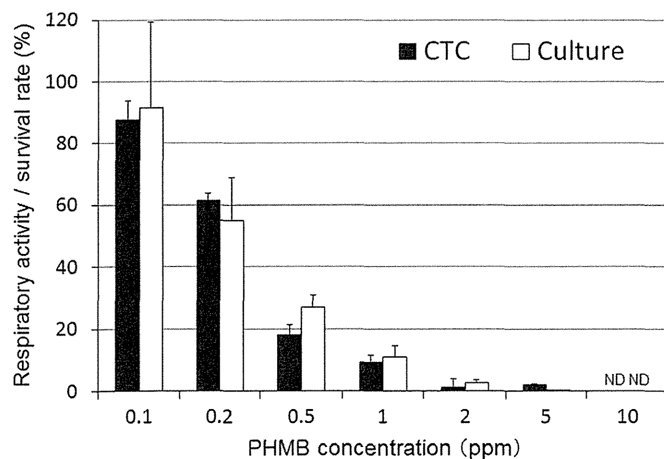

FIG 4 Respiratory activity and survival rate of *Acanthamoeba* trophozoites after PHMB treatment. The respiratory activity (CTC biocidal assay) was compared with the survival rate (culture-dependent biocidal assay) for trophozoites after treatment with PHMB (25°C, 4 h). Error bars represent the standard errors of the means for four experiments. ND, not detected (<1%).

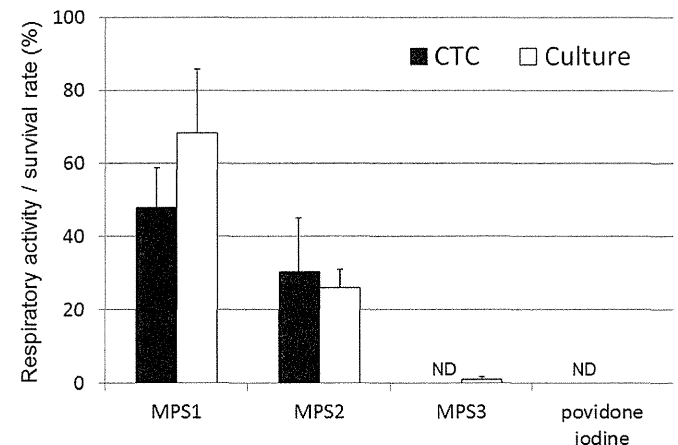

FIG 5 Respiratory activity and survival rate of *Acanthamoeba* trophozoites after treatment with SCL disinfectant solutions. The respiratory activity (CTC biocidal assay) was compared with the survival rate (culture-dependent biocidal assay) for trophozoites after treatment with SCL disinfectant solutions (25°C, 4 h). Error bars represent the standard errors of the means for four experiments. ND, not detected (<1%).

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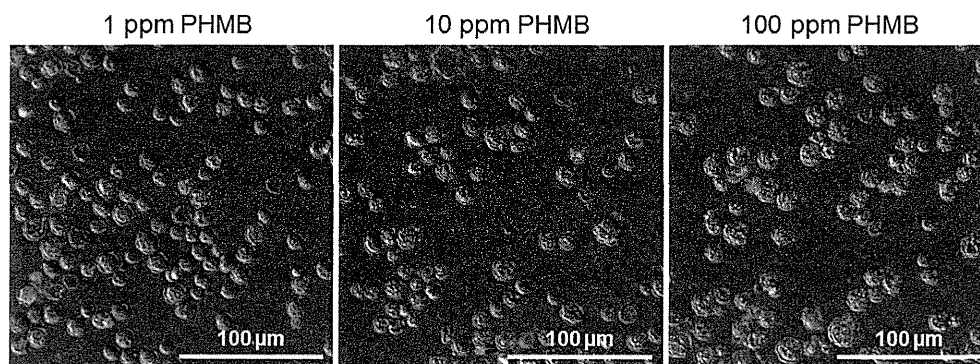

FIG 6 CTC staining of PHMB-treated *Acanthamoeba* cysts. Merged fluorescence images of red-fluorescing CTC formazan and differential interference contrast images of cysts stained with CTC after PHMB treatments are shown. CTC staining was performed on cysts treated with PHMB (1, 10, or 100 ppm) for 4 h at 25°C.

respectively. There was a significant positive correlation between the respiratory activity (CTC biocidal assay) and the survival rate (culture-dependent biocidal assay) for the trophozoites treated with SCL disinfectant solutions ($r = 0.70$ and $P < 0.01$; Spearman rank correlation test).

The fluorescence signals from cysts that were stained with CTC after the PHMB treatments were decreased in proportion to the PHMB concentration (Fig. 6). The cysts exposed to 1 ppm of PHMB appeared red by fluorescence, indicating that most of the cysts maintained their respiratory activity. A weak fluorescence was observed after exposure of cysts to 10 ppm or 100 ppm PHMB, although this was partially due to autofluorescence of the cysts.

The respiratory activities and survival rates of the cysts after the PHMB treatments (4 h) are shown in Fig. 7. The respiratory activities were 37.6%, 1.5%, 1%, and 0% of the control level and the survival rates were 30.3%, 0.4%, 0.01%, and 0% of the control level for cysts treated with 1, 10, 100, and 300 ppm of PHMB, respectively. Thus, the respiratory activity (CTC biocidal assay) and survival rate (culture-dependent biocidal assay) were reduced after PHMB treatment, in a dose-dependent manner. A significant positive correlation between the respiratory activity and the survival rate was present for the cysts treated with PHMB ($r = 0.60$ and $P < 0.01$; Spearman rank correlation test).

The respiratory activities and survival rates of the cysts after a 4-h exposure to SCL disinfectant solutions are shown in Fig. 8. The respiratory activities were 9.8%, 24.4%, 11.0%, and 1.5% of the control level and the survival rates were 40.5%, 54.9%, 22.8%, and 4.4% of the control level for the cysts treated with MPS1, MPS2, MPS3, and povidone iodine solution, respectively. There was a significant positive correlation between the respiratory activity (CTC biocidal assay) and the survival rate (culture-dependent biocidal assay) for cysts treated with SCL disinfectant solutions ($r = 0.64$ and $P < 0.01$; Spearman rank correlation test).

DISCUSSION

Our CTC staining results showed that the red fluorescent compound formazan, which represents the respiratory activity of *Acanthamoeba* organisms, accumulated in trophozoites and cysts. The CTC biocidal assay demonstrated clearly that the respiratory activity of *Acanthamoeba* trophozoites and cysts was decreased in a dose-dependent way after exposure to PHMB.

Conventional culture-dependent methods have been used to evaluate the effectiveness of various disinfectants against *Acan-*

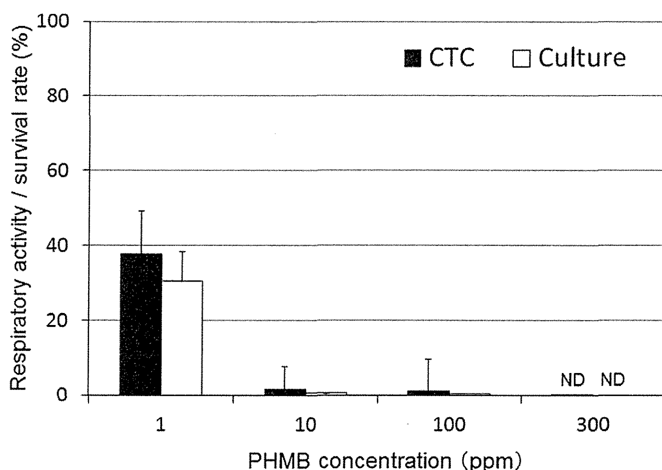

FIG 7 Respiratory activity and survival rate of *Acanthamoeba* cysts after PHMB treatment. The respiratory activity (CTC biocidal assay) was compared with the survival rate (culture-dependent biocidal assay) for cysts after treatment with PHMB (25°C, 4 h). Error bars represent the standard errors of the means for four experiments. ND, not detected (<1%).

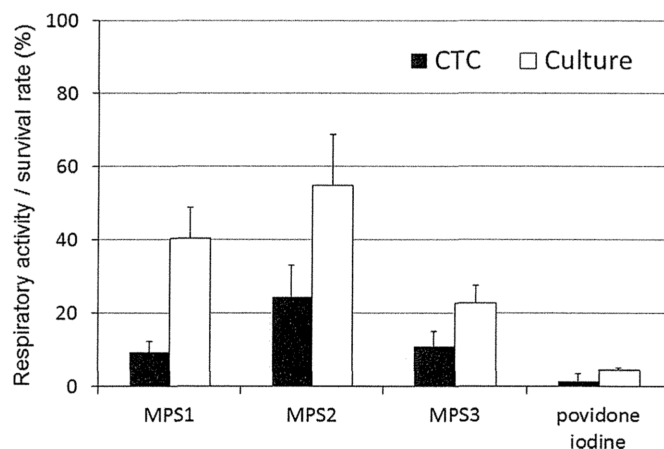

FIG 8 Respiratory activity and survival rate of *Acanthamoeba* cysts after treatment with SCL disinfectant solutions. The respiratory activity (CTC biocidal assay) was compared with the survival rate (culture-dependent biocidal assay) for cysts after treatment with SCL disinfectant solutions (25°C, 4 h). Error bars represent the standard errors of the means for four experiments.

thamoeba (1, 2, 11, 12, 15, 20, 24). Among these, methods using the MPN method or the Spearman-Karber method have been considered suitable methods to quantify the number of living organisms (1, 18, 24) because they are simple, reliable, and reproducible for standardized efficacy tests (1, 18).

Our results showed that the respiratory activity determined by the CTC biocidal assay was significantly correlated with the survival rate determined by culture-dependent biocidal assay for both trophozoites and cysts treated with PHMB and SCL disinfectants. These results indicate that the respiratory activities determined by the CTC biocidal assay are strongly correlated with the number of living *Acanthamoeba* organisms. It has been well documented that CTC is a good estimator of bacterial viability, and thus the CTC biocidal assay can be used as an alternative method to conventional culture-dependent methods to assay the number of living *Acanthamoeba* organisms.

Although the culture-dependent biocidal assay has been accepted as an efficient assay to test the disinfecting properties of anti-*Acanthamoeba* solutions, detection of surviving *Acanthamoeba* organisms requires 1 to 3 weeks of cultivation for trophozoites and cysts, respectively. On the other hand, the CTC biocidal assay requires only about 2 h for *Acanthamoeba* trophozoites and 18 h for cysts. The CTC biocidal assay requires 30 min for staining and 30 min for fixation following the disinfectant treatment, and it also requires 16 h of preincubation before CTC staining for cysts. In addition, by using a fluorescence microplate reader to measure the fluorescence intensity of CTC-stained samples, we were able to analyze multiple samples in a very short time. Although a good correlation was found between the two assay methods within the range of about 2 log units (>1%), the range of sensitivity of the CTC biocidal assay is about 2 log units, while the range of sensitivity of the culture-dependent method is 3 log units or more. Therefore, the CTC biocidal assay can be used for rapid testing and screening of new disinfectants, and the culture method might be necessary to confirm the final results.

However, unstained *Acanthamoeba* organisms also have weak autofluorescence, and the autofluorescence levels varied after either trophozoites or cysts were treated with different disinfectants (data not shown). To overcome this problem, the samples that had sodium azide added to inhibit respiration were used as negative controls. Sodium azide is known to inhibit the respiratory activity of bacteria (28), and it has been reported that exposure to sodium azide (2 mg/ml) inhibits CTC reduction by protozoa, while sodium azide does not affect autofluorescence (14). Thus, to normalize the fluorescence intensity, the fluorescence intensity of the negative control was subtracted from the value of the test sample.

The efficacies of other rapid staining methods for detecting living or dead *Acanthamoeba* organisms have been examined (3, 16). Propidium iodide (PI) penetrates cells with damaged membranes and binds to DNA, and it also stains dead cells (21). Although its effectiveness has been correlated with that of methylene blue staining (3), a significant correlation has never been reported because of the difficulty in estimating the number of living cells by staining dead cells. Fluorescein diacetate (FDA), on the other hand, is hydrolyzed by intracellular esterases and stains live cells (8). However, it also stains dead cells because of the presence of residual esterase activity (4, 5). Thus, this method overestimates the number of living amoebae (16). These shortcomings are overcome by the CTC biocidal assay.

There is a concern about the preincubation step for *Acanthamoeba* cysts to restore respiratory activity. In our preliminary experiments, no fluorescence signal of CTC formazan was detected in the cysts after CTC staining because the cysts were dormant and may have had little or no respiratory activity. CTC formazan accumulation was observed in most of the cysts after 16 h of preincubation, indicating that 16 h of preincubation was necessary to restore the respiratory activity of *Acanthamoeba* cysts. In addition, the 16-h preincubation period did not lead to any proliferation of *Acanthamoeba*. Thus, 16 h of preincubation is an appropriate duration for *Acanthamoeba* cysts in the CTC biocidal assay. The strong correlation between the results of the CTC biocidal assay and those of the culture-dependent biocidal assay suggests that the results determined by CTC biocidal assay most likely represent the number of living *Acanthamoeba* cysts, although the results may not reflect the exact respiratory activity of *Acanthamoeba* cysts.

The respiratory activities of cysts treated with SCL disinfectant solutions tended to be lower than the survival rates. This suggests that the recovery of respiratory activity is delayed in living cysts. A difference in encystment rates between different SCL disinfectant solutions has been described because of the different ingredients in SCL disinfectant solutions (20). Thus, our results suggest that the ingredients in SCL disinfectant solutions may affect the recovery of respiratory activity in cysts, and the preincubation time may be different for each SCL disinfectant solution. A further optimization of the preincubation time might be necessary for each SCL disinfectant solution for CTC biocidal assay for cysts.

In conclusion, CTC staining can be used to detect the respiratory activity of *Acanthamoeba* trophozoites and cysts, and the CTC biocidal assay can be a rapid and simple method to assay the effectiveness of a disinfectant agent against *Acanthamoeba*.

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REFERENCES

1. **Beattie TK, Seal DV, Tomlinson A, McFadyen AK, Grimason AM.** 2003. Determination of amoebicidal activities of multipurpose contact lens solutions by using a most probable number enumeration technique. J. Clin. Microbiol. **41**:2992–3000.
2. **Borazjani RN, Kilvington S.** 2005. Efficacy of multipurpose solutions against Acanthamoeba species. Cont. Lens Anterior Eye **28**:169–175.
3. **Borazjani RN, May LL, Noble JA, Avery SV, Ahearn DG.** 2000. Flow cytometry for determination of the efficacy of contact lens disinfecting solutions against Acanthamoeba spp. Appl. Environ. Microbiol. **66**:1057–1061.
4. **Breeuwer P, Drocourt JL, Rombouts FM, Abee T.** 1994. Energy-dependent, carrier-mediated extrusion of carboxyfluorescein from Saccharomyces cerevisiae allows rapid assessment of cell viability by flow cytometry. Appl. Environ. Microbiol. **60**:1467–1472.
5. **Catala P, et al.** 1999. Effectiveness of CSE to counterstain particles and dead bacterial cells with permeabilised membranes: application to viability assessment in waters. FEMS Microbiol. Lett. **178**:219–226.
6. **Centers for Disease Control and Prevention.** 2007. Acanthamoeba keratitis multiple states, 2005–2007. MMWR Morb. Mortal. Wkly. Rep. **56**: 532–534.
7. **Creach V, Baudoux AC, Bertru G, Rouzic BL.** 2003. Direct estimate of active bacteria: CTC use and limitations. J. Microbiol. Methods **52**:19–28.
8. **Diaper JP, Edwards C.** 1994. Survival of Staphylococcus aureus in lakewater monitored by flow cytometry. Microbiology **140**:35–42.
9. **Gray TB, Cursons RT, Sherwan JF, Rose PR.** 1995. Acanthamoeba, bacterial, and fungal contamination of contact lens storage cases. Br. J. Ophthalmol. **79**:601–605.
10. **Hamilton M, Russo R, Thurston R.** 1977. Trimmed Speaman-Karber

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method for estimating median lethal concentrations in toxicity bioassays. Environ. Sci. Technol. 11:714–719.
11. **Hiti K, Walochnik J, Haller-Schober EM, Faschinger C, Aspock H.** 2002. Viability of Acanthamoeba after exposure to a multipurpose disinfecting contact lens solution and two hydrogen peroxide systems. Br. J. Ophthalmol. **86**:144–146.
12. **Hugo ER, McLaughlin WR, OH KH, Tuovinen OH.** 1991. Quantitative enumeration of Acanthamoeba for evaluation of cyst inactivation in contact lens care solutions. Invest. Ophthalmol. Vis. Sci. 32:655–657.
13. **Illingworth CD, Cook SD.** 1998. Acanthamoeba keratitis. Surv. Ophthalmol. 42:493–508.
14. **Iturriaga R, Zhang S, Sonek GJ, Stibbs H.** 2001. Detection of respiratory enzyme activity in Giardia cysts and Cryptosporidium oocysts using redox dyes and immunofluorescence techniques. J. Microbiol. Methods **46**:19–28.
15. **Johnston SP, et al.** 2009. Resistance of Acanthamoeba cysts to disinfection in multiple contact lens solutions. J. Clin. Microbiol. **47**:2040–2045.
16. **Khunkitti W, Avery SV, Lloyd D, Furr JR, Russell AD.** 1997. Effects of biocides on Acanthamoeba castellanii as measured by flow cytometry and plaque assay. J. Antimicrob. Chemother. **40**:227–233.
17. **Kilvington S, et al.** 2004. Acanthamoeba keratitis: the role of domestic tap water contamination in the United Kingdom. Invest. Ophthalmol. Vis. Sci. **45**:165–169.
18. **Kobayashi T, et al.** 2011. Efficacy of commercial soft contact lens disinfectant solutions against Acanthamoeba. Jpn. J. Ophthalmol. **55**:547–557.
19. **Larkin DF, Kilvington S, Easty DL.** 1990. Contamination of contact lens storage cases by Acanthamoeba and bacteria. Br. J. Ophthalmol. **74**:133–135.
20. **Lonnen J, Heaselgrave W, Nomachi M, Mori O, Santodomingo-Rubido J.** 2010. Disinfection efficacy and encystment rate of soft contact lens multipurpose solutions against Acanthamoeba. Eye Contact Lens **36**:26–32.
21. **Lopez-Amoros R, Comas J, Vives-Rego J.** 1995. Flow cytometric assessment of Escherichia coli and Salmonella typhimurium starvation-survival in seawater using rhodamine 123, propidium iodide, and oxonol. Appl. Environ. Microbiol. **61**:2521–2526.
22. **Marciano-Cabral F, Cabral G.** 2003. Acanthamoeba spp. as agents of disease in humans. Clin. Microbiol. Rev. **16**:273–307.
23. **Neff R, Ray S, Benton W, Wilborn M.** 1964. Induction of synchronous encystment (differentiation) in Acanthamoeba sp. Methods Cell Physiol. 1:55–83.
24. **Perrine D, et al.** 1995. Amoebicidal efficiencies of various diamidines against two strains of Acanthamoeba polyphaga. Antimicrob. Agents Chemother. 39:339–342.
25. **Pyle BH, Broadaway SC, McFeters GA.** 1995. Factors affecting the determination of respiratory activity on the basis of cyanoditolyl tetrazolium chloride reduction with membrane filtration. Appl. Environ. Microbiol. **61**:4304–4309.
26. **Rodriguez GG, Phipps D, Ishiguro K, Ridgway HF.** 1992. Use of a fluorescent redox probe for direct visualization of actively respiring bacteria. Appl. Environ. Microbiol. **58**:1801–1808.
27. **Smith JJ, Howington JP, McFeters GA.** 1994. Survival, physiological response and recovery of enteric bacteria exposed to a polar marine environment. Appl. Environ. Microbiol. **60**:2977–2984.
28. **Smith JJ, McFeters GA.** 1997. Mechanisms of INT (2-(4-iodo-phenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride), and CTC (5-cyano-2,3-ditolyl-tetrazolium chloride) reduction in Escherichia coli K-12. J. Microbiol. Methods **29**:161–175.
29. **Thebpatiphat N, et al.** 2007. Acanthamoeba keratitis: a parasite on the rise. Cornea **26**:701–706.
30. **Winding A, Binnerup SJ, Sorensen J.** 1994. Viability of indigenous soil bacteria assayed by respiratory activity and growth. Appl. Environ. Microbiol. **60**:2869–2875.

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Antimicrobial Efficacy Tests of Multipurpose Contact Lens Care Solutions Simulating Poor Contact Lens Hygiene Behaviors

Toshihiko Uno, M.D., Ph.D., Yuichi Ohashi, M.D., Ph.D., and Masaki Imayasu, Ph.D.

Purpose: The aim of this study was to simulate the biofilm formation in contact lens (CL) case under poor hygiene behaviors; antimicrobial efficacies of multipurpose solutions (MPSs) against biofilm on the lens case were evaluated.

Methods: Five MPSs (Epica Cold, Complete 10 min, ReNu MultiPlus, SoftOne Mois, and OPTI-FREE Plus) were tested. Lens cases containing ACUVUE2 were inoculated with 1×10^1, 10^3, or 10^6 colony-forming units (CFUs) of *Staphylococcus epidermidis* (SE). Each lens case was treated with 1 MPS for 4 hrs followed by the estimation of the number of SE by the CFU method. Disinfection efficacies of MPSs against SE biofilm were evaluated by biomicroscopy with safranin staining and scanning electron microscopy.

Results: Lens cases, inoculated with 1×10^1 CFU, were disinfected by all MPSs. Epica Cold, Complete 10 min, ReNu MultiPlus, and OPTI-FREE showed almost a 2-log reduction of the CFU, whereas SoftOne Mois effect was almost a 1 log reduction, significantly lower than other MPSs ($P<0.05$). No biofilm formations were observed in Epica Cold, Complete 10 min, ReNu MultiPlus, and OPTI-FREE Plus-treated groups unlike significant biofilm formation in the SoftOne Mois-treated group ($P<0.01$).

Conclusions: Greater efforts to educate patients regarding compliant lens care behavior are needed to reduce the incidence of CL-associated microbial keratitis.

Key Words: MPS—Biofilm—SEM—*Staphylococcus epidermidis*.

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INTRODUCTION

Recently, an outbreak of *Fusarium* keratitis, a rare fungal infection, has been linked to contact lens (CL) wear in Singapore,[1,2] Hong Kong,[3] and the United States.[4] This fungal epidemic among CL wearers strongly implicated one specific multipurpose solution (MPS), ReNu with Moisture Loc.[4] This outbreak is believed to be caused by insufficient antimicrobial efficacy of this MPS against *Fusarium solani* (FS) after evaporation.[5] Furthermore, Verani,[6] a researcher at the Center for Disease Control has presented in the Food and Drug Administration panel meeting in 2008 that noncompliant lens care behaviors such as "topping off" or reuse of MPS caused a 4.4-fold higher risk of microbial keratitis. These findings clearly showed a direct correlation between SCL-related microbial keratitis and noncompliant lens care behaviors.

On the other hand, it is well known that "biofilm," extracellular sugar–protein secreted by bacteria, increases the affinity of bacterial colonies to biomaterial surfaces and also enhances the tolerance to antibiotics 1,000 times higher than their planktonic cells.[7] It was also suggested that biofilm formation of infectious bacteria may be associated with high incidence of microbial keratitis.[8] Investigations over the last two decades have shown that mechanical rubbing and subsequent rinsing of CL are two important key elements to prevent biofilm formation on the surface of CL and lens storage cases.[9–11] For example, Sutton et al.[11] demonstrated that a combination of mechanical rubbing and rinsing of CL reduced the level of biofilm to 10^{-3} to 10^{-5} using the multiitem microbial challenge test in vitro.

Pseudomonas aeruginosa (PA) and coagulase-negative *Staphylococci* (CNS) have been historically associated with soft CL (SCL)-related infection keratitis.[12] Among these microorganisms, *Staphylococcus epidermidis* (SE), a typical CNS, is known to be the highest biofilm producer.[13] Wu et al.[14] reported that CNS was the most frequently recovered microorganisms contaminated in CL storage cases. In addition, the intercellular adhesion genes icaA has been shown to be expressed only in biofilm-forming SE strains.[13] Suzuki et al.[8] have shown that icaA gene expression was 60% higher in the conjunctiva-isolated SE compared with facial skin-isolated SE. In view of these findings, it is believed that conjunctiva-isolated SE might be responsible for bacterial keratitis seen among CL wearers.

In this study, we investigated the efficacy of five commercially available MPSs in simulated poor lens care behaviors using three test methods. First, antimicrobial efficacies of test MPSs against SE strain, a model microorganism forming biofilm, at three different inoculation size were evaluated in a lens storage case containing an SCL. Second, the formation of SE biofilm in lens storage cases containing an SCL was evaluated after the cycle treatment with test MPS. Third, the breakdown of SE biofilm treated with test MPS was observed with scanning electron microscopy (SEM).

MATERIALS AND METHODS

Multipurpose Solutions

Five different kinds of MPS were purchased from commercial sources and were used before their expiration date. The ingredients of these MPSs are shown in Table 1.

Staphylococcus epidermidis Culture

The SE (biofilm-forming clinical strain, PAGU255) was a gift from Prof. Yoshiaki Kawamura of the Department of Microbiology

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TABLE 1. *Multipurpose Solutions Tested*

MPS	Manufacturer	Preservative	Surfactant	Buffer
Epica cold	Menicon	PHMB (1.0 ppm)	HCO	None
Complete 10 min	Advanced Medical Optics	PHMB (1.0 ppm)	Poloxamer	Phosphate
ReNu MultiPlus	Bausch & Lomb	PHMB (1.1 ppm)	Poloxamine	Boric acid
SoftOne Mois (version g)	Rohto	PHMB (1.0 ppm)	Poloxamer	Phosphate
OPTI-FREE Plus	Alcon	POLYQUAD (10 ppm)	Poloxamine	Boric acid

HCO, macrogolglycerol hydroxystearate; PHMB, polyhexamethylene biguanide.

at the Aichigakuin University in Nagoya, Japan. The strain was maintained in Soybean–Casein Digest Agar (SCDA, Nihon-Seiyaku, Tokyo, Japan) plates at 4°C. Bacteria were cultured in SCDA for 24 hrs at 32°C. The concentration of bacteria was adjusted turbidometrically using a spectrophotometer (BPM-20, Taitec, Saitama, Japan) to an optical density of 0.1 at 660 nm, which is approximately equivalent to 1×10^8 colony-forming units (CFU) per milliliter and stored in a refrigerator. A part of the bacterial suspension was diluted 10^{-6} fold, and 1 mL of the diluted sample was inoculated into SCDA at 32°C. After 24 hrs, the number of colonies formed was counted. Then, the CFU of the original bacterial suspension was accurately adjusted to 1×10^8 CFU per milliliter. Bacterial saline dilutions of 1×10^5 or 1×10^3 CFU per milliliter were used in subsequent experiments.

Antimicrobial Efficacy of MPS With Lens Storage Case Containing SCL

Soft Contact Lenses (ACUVUE2, base curve: 8.3 mm, power: −3.00 D, diameter: 14.0 mm, Johnson & Johnson, Jacksonville, FL) were presoaked in saline overnight. These lenses were immersed in 1 mL of fetal bovine serum (Invitrogen, Grand Island, NY) for 5 sec at room temperature. Organic soiling with bovine serum is recommended for antimicrobial activity tests.[15,16] Soiled SCLs were placed into each brand of lens storage case convex side up. Ten microliters of 3 different concentrations of bacterial suspension (1×10^1, 1×10^3, and 1×10^6 CFU per lens) were inoculated into soiled SCLs for 10 min. Two milliliters of each MPS was added to the bacterial suspensions in soiled SCLs placed in lens storage cases. After 4 hrs of incubation at 22°C, the lens storage cases were sonicated for 5 min and vortexed. One milliliter of bacterial suspensions in each SCL from lens storage cases was collected with 10 mL of Dey and Engley (D/E) neutralizing broth (DIFCO, Sparks, MD) and inoculated onto SCDA. After 5 days of incubation at 37°C, the number of colonies formed was calculated. The 3 different concentrations of 1×10^1, 1×10^3, and 1×10^6 CFU per lens bacteria used correspond to the estimated number of bacteria in a typical lens storage case, after mechanical rubbing, and after subsequent rinsing, respectively.[12]

Biofilm Formation in Lens Storage Cases

The treatment cycle described above (lens soiling with serum—bacterial inoculation—incubation for 4 hrs with MPS) was repeated 3 times without changing MPSs. After washing the lens storage case with saline, the inner surfaces were stained with 0.2% safranin for biofilm formation analysis. Safranin was generally used for staining biofilms of CNS.[17] The stained lens storage cases were photographed with a digital camera (TIF files) followed by image analysis for the percentage of safranin-staining area per each well of lens case. Six wells in three lens cases were analyzed for each test solution.

Scanning Electron Microscopy of Biofilm Formation and Removal

For SEM analysis, a suspension of SE (1×10^5 per well) was inoculated into lens storage cases. The cases were incubated overnight at 32°C and gently washed with saline. Each case was treated with 2 mL of 1 of the 5 kinds of MPSs at room temperature for 4 hrs. After removing the MPSs and gently washing the cases with saline, lens cases with bacterial cells were fixed with 2.5% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA) in 1% 100 mM sodium cacodylate buffer (pH 7.4) for 1 hr at 4°C, and then postfixed with 1% Osmium tetroxide (OsO_4) in the same buffer for 1 hr at 4°C. After successive graded ethanol (70, 80, 85, 90, 95, 100%) dehydration, the specimens were dried by the critical point method (HCD-1, Hitachi, Tokyo, Japan). Dried specimens were mounted on stubs with conductive adhesive tape and conventionally coated with a thin layer of palladium–platinum in

TABLE 2. *Total Counts of SE in the Lens Storage Cases (CFU per Lens)*

MPS	Inoculation Size	Test 1	Test 2	Test 3	Average
Epica Cold	1×10^6	5.7×10^3	1.21×10^4	8.0×10^3	8.6×10^3
	1×10^3	0	4.0×10^1	1.0×10^1	1.7×10^1
	1×10^1	0	0	0	0
Complete 10 min	1×10^6	8.9×10^3	4.6×10^3	1.28×10^4	8.8×10^3
	1×10^3	0	4.0×10^1	1.0×10^1	1.7×10^1
	1×10^1	0	0	0	0
ReNu MultiPlus	1×10^6	1.9×10^3	5.4×10^3	5.5×10^3	4.3×10^3
	1×10^3	0	0	5.0×10^1	1.7×10^1
	1×10^1	0	0	0	0
SoftOne Mois	1×10^6	4.2×10^4	4.2×10^4	7.2×10^4	5.2×10^4
	1×10^3	1.4×10^2	1.6×10^2	9.0×10^1	1.3×10^2
	1×10^1	0	0	0	0
OPTI-FREE Plus	1×10^6	4.5×10^3	1.9×10^3	4.9×10^3	3.8×10^3
	1×10^3	0	2.0×10^1	4.0×10^1	2.0×10^1
	1×10^1	0	0	0	0

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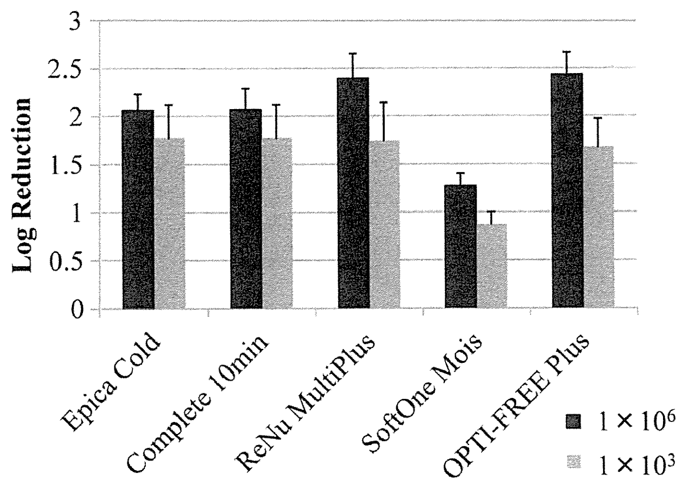

FIG. 1. The results of multipurpose solution (MPS) antimicrobial efficacy tests using ACUVUE2 lenses in contact lens cases. Final concentrations of the inoculants were 1×10^3 and 1×10^6 CFU per lens.

a sputter coater (SC500, Meiwa, Osaka, Japan). The specimens were viewed with a field-emission scanning electron microscope (S4800, Hitachi, Tokyo, Japan) at 5 kV followed by image analysis for the percentage of biofilm-formed area per each SEM image (TIF files). Four representative images were analyzed for each test solution.

Image Analysis

The TIF color files were converted into 16-bit gray TIF files using Adobe Photoshop (Adobe System Inc., San Jose, CA). The gray files were converted into binary file at appropriate scale as the threshold followed by determination of restricted area, showing significant safranin staining or biofilm formation, using ImageJ software (developed by Wayne Rasband, National Institute of Health, Bethesda, MD).

Statistical Analyses

Statistical analyses of the value of log reduction, safranin-staining area, and biofilm-formed area were performed using analysis of variance with the Scheffe test by means of Excel Stat (Microsoft, Redmond, WA).

RESULTS

Antimicrobial Efficacies of MPSs With Lens Storage Cases Containing SCL

Table 2 shows the results for the MPSs antimicrobial efficacies against SE cultured on test SCL in lens storage cases. All 5 MPSs killed 1.0×10^1 CFU of SE after 4 hrs. Epica Cold, Complete 10 min, ReNu MultiPlus and OPTI-FREE Plus reduced SE colony from 1.0×10^3 CFU to 1.7×10^1, 1.7×10^1, 1.7×10^1, and 2.0×10^1 CFU, respectively. SoftOne Mois demonstrated a weak antimicrobial effect with 1.3×10^2 CFU of SE after 4 hrs of treatment. Similar tendencies were also observed with 1.0×10^6 CFU of SE. Epica Cold, Complete 10 min, ReNu MultiPlus and OPTI-FREE Plus reduced the initial 1.0×10^6 CFU of SE to 8.6×10^3, 8.8×10^3, 4.3×10^3, and 3.8×10^3 CFU, respectively. SoftOne Mois showed weak antimicrobial effect again with 5.2×10^4 CFU of SE after 4 hrs of treatment.

Figure 1 shows log reduction of SE after 4 hrs of treatment with each MPS. Epica Cold, Complete 10 min, ReNu MultiPlus, and OPTI-FREE demonstrated almost a 2-log reduction of the CFU of SE, whereas SoftOne Mois effect was almost a 1 log reduction, which was significantly lower than other 4 MPSs ($P<0.05$, analysis of variance [ANOVA] with Scheffe).

Biofilm Formation in Lens Storage Cases After Cycle Treatment With MPS

Figure 2(A–E) shows photographs of MPSs-treated lens storage cases. There were no biofilm formation in Epica Cold, Complete

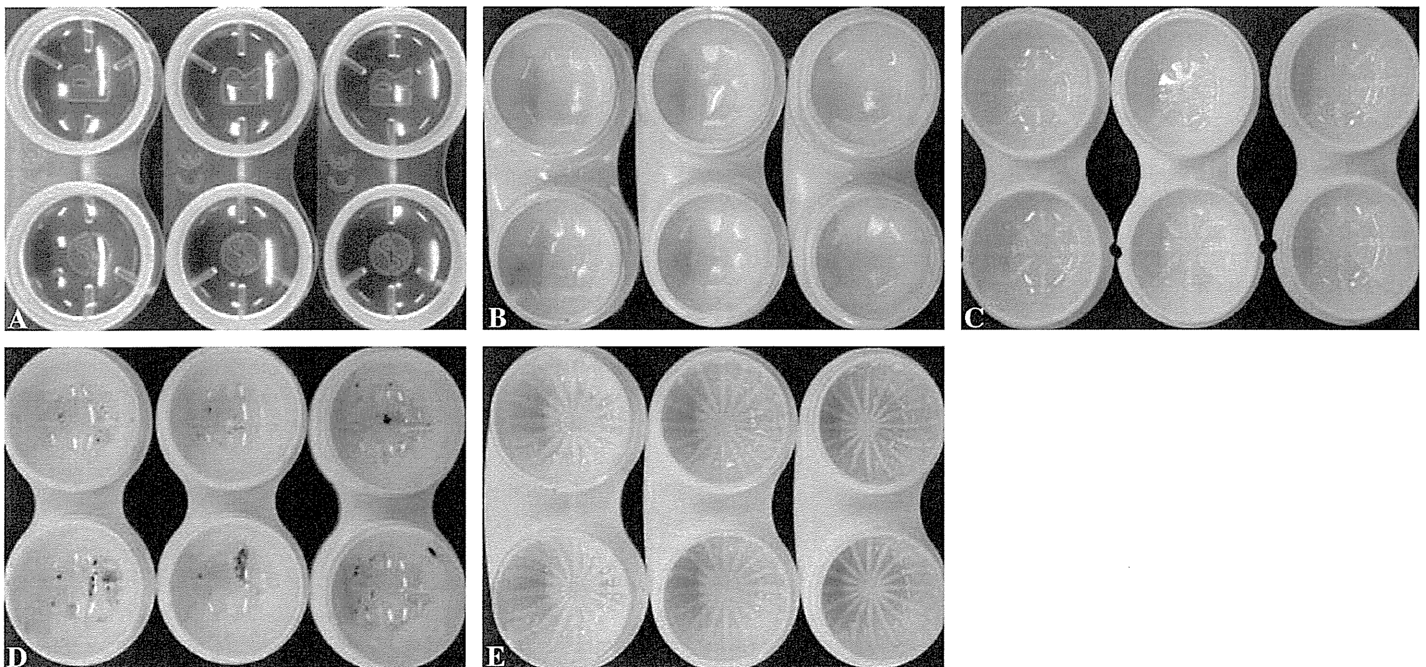

FIG. 2. View of each brand of lens storage case after 3 treatment cycles with each MPS. (A) Epica Cold; (B) Complete 10 min; (C) ReNu MultiPlus; (D) SoftOne Mois; (E) OPTI-FREE Plus.

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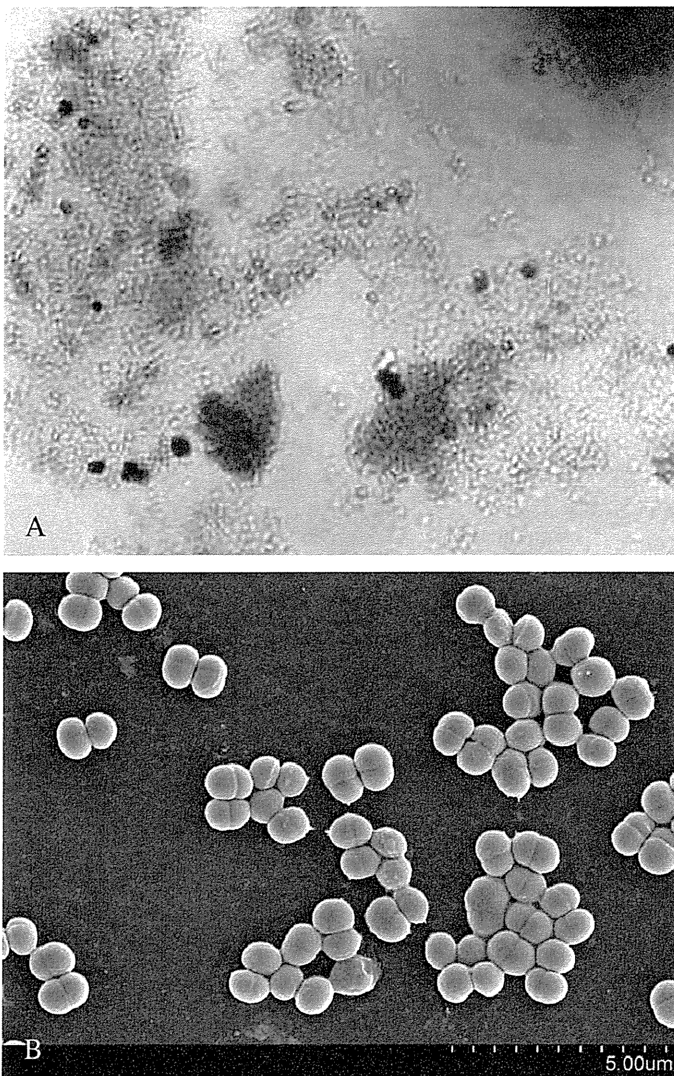

FIG. 3. Light and scanning electron microscopy (SEM) views of a lens storage case after 3 treatment cycles with SoftOne Mois. (A) Light micrograph (original magnification ×400); (B) SEM image (original magnification ×8,000).

10 min, ReNu MultiPlus, and OPTI-FREE Plus-treated lens storage cases (Fig. 2A–C and E). However, a biofilm formation was observed in the SoftOne Mois-treated lens storage case (Fig. 2D). Figure 3(A and B) shows microphotograph and SEM images of the SoftOne Mois-treated. The SEM data revealed a biofilm composed of cocci of SE. Figure 4 shows the results of image analysis of safranin-staining area. SoftOne Mois-treated lens storage case showed the highest safranin-staining area, 0.43%±0.09%, which was significantly higher than other lens cases ($P<0.01$, ANOVA with Scheffe).

Effect of MPSs on Biofilm Removal in Lens Storage Cases

The SEM was used to evaluate the capacity of each MPS to remove SE biofilm. Figure 5(A–E) shows the SEM images of MPSs-treated lens storage cases. The biofilm areas were reduced by treatment with Epica Cold, Complete 10 min and ReNu MultiPlus (Fig. 5A–C) compared with SoftOne Mois, OPTI-FREE Plus, and saline control (Fig. 5D–F); however, no significant differences

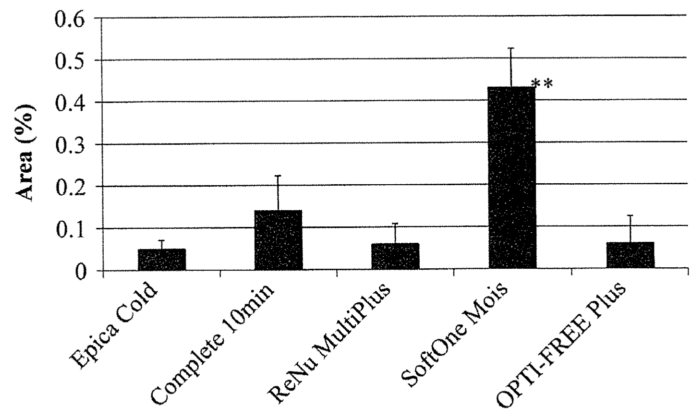

FIG. 4. The results of image analysis of safranin-staining area in a lens storage case. The percentage of safranin-staining area per each well of lens case was analyzed by image analysis. The error bars are SD obtained from six separate lens cases.

were shown among these 6 groups because of high standard deviations as shown in Figure 6.

DISCUSSION

Generally, the antimicrobial efficacies of MPSs are evaluated using the stand-alone test procedure described in ISO14729 guidelines.[15] For example, the stand-alone test was used to determine the efficacy of MPSs on a standard inoculum of a representative range of microorganisms (PA, *Staphylococcus aureus*, *Serratia marcescens*) and two fungi (*Candida albicans*, FS).[15] In our preliminary study, we evaluated the antimicrobial efficacy of all five MPSs on a non-ISO standard strain of SE. All tested MPSs achieved a 5-log reduction in SE viability. Practically, it is impossible to achieve such a high killing efficiency because some microorganisms, such as SE, develop resistance to disinfectants through biofilms formation.[18]

McLaughlin-Borlace et al.[18] have investigated bacterial biofilm formation from SCL and lens storage cases from 20 SCL wearers with microbial keratitis using SEM technique and showed that bacterial population analysis from biofilms and cornea from these 20 patients were similar and mostly composed of rods bacteria, cocci bacteria, fungi, and amoeba cysts. Garcia-Saenz et al.[19] showed that an SE biofilm producer ATCC35984 strain exhibited higher adhesion to SCL than did an SE non–biofilm-producer ATCC12228 strain. Similarly, Gabriel et al.[20] demonstrated that a slime producer #230022B SE strain showed a higher retention rate compared with a non–slime-producer #15072 to SCLs. These findings showed that biofilm-forming bacteria can develop resistance against MPSs that have been shown to have a 5-log reduction in bacterial number in the ISO stand-alone test.

Several investigations have shown that mechanical rubbing and subsequent rinsing of CL are keys to microbial keratitis prevention.[9–11] Wu et al.[21] also showed that mechanical rubbing and wiping of the lens case were the most effective cleaning regimen in reducing biofilm. Contact lens rubbing has been shown to reduce the CFU by a 1 to 2 log, whereas rinsing has been shown to reduce the colony-forming units CFU by a 3 to 5 log.[11] The complete disinfection at 1×10^1 CFU and absence of disinfection at 1×10^3 or 10^6 CFU of MPSs in this study showed that MPS antimicrobial efficacies are dependent on initial SE density. It is interesting to

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