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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Effects of multi-purpose contact-lens care solutions on the adhesion of *Acanthamoeba* to silicone hydrogel contact lenses

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Abstract

Purpose: To evaluate the effect of seven multi-purpose contact-lens care solutions (MPSs) on the adhesion of *Acanthamoeba* (AC) to five 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 hours incubation, the number of adherent AC trophozoites on SHCL was counted under phase-contrast microscopy. AC trophozoites mixed with seven 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 to the other three 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 Mois was about 50% and 75% of control, respectively. Normal AC trophozoites without any treatments showed 25 times higher adhesion rates compared to 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.

Keywords: Acanthamoeba, contact lens, multi-purpose contact-lens care solution

INTRODUCTION

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 difficult diagnosis 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 lenses (SCL) wear, and AC infection occurs through poor lens hygiene practices with non compliant behaviors.[4,5] AC has two 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 (SHCL) with higher oxygen transmissibility were introduced to the world contact lens market nearly a decade ago. Studies have found no significant difference in microbial keratitis incidence associated with SHCL and conventional SCLs wearing.[7,8] However, recent studies have shown that AC has a higher affinity to SHCLs, Lotrafilcon A and Balafilcon A, in comparison to 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.

On the other hand, most contact lens wearers utilize multi-purpose 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.

glucose (PYG) medium (Bacto Proteose Peptone 20.02g and Yeast Extract 1.00g in 950mL pure water, 2M D(+)Glucose 50.0mL, 0.4M $MgSO_4$-$7H_2O$ 10.0mL, 0.05M $CaCl_2$ 8.0mL, 0.1M Sodium Citrate-$2H_2O$ 34.0mL, 0.005M $(Fe(NH_4)_2(SO_4)_2$-$6H_2O$ 10.0mL, 0.25M $NaHPO_4$-$7H_2O$ 10.0mL, 0.25M KH_2PO_4 10.0mL) 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 (NaCl 2.15g, KCl 0.075g, $CaCl_2$-$2H_2O$ 0.076g, 1000mL pure water) twice by centrifugation at 300G (EX-125, TOMY, Tokyo, Japan) and resuspended in fresh 1/4 strength Ringer solution. The AC cysts were prepared by incubation in encystment medium (NaCl 14.61g, $MgCl_2$-$6H_2O$ 0.651g, $CaCl_2$-$2H_2O$ 0.053g, 1000mL 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 1×10^5cells/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. Fifty (50) μL of trophozoites suspension 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 as well.

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

Multi-Purpose 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, because of its characterization by many investigators.[11-15] AC trophozoites and cysts were prepared as described previously.[17] Briefly, AC trophozoites were axenically cultured in a culture flask (BD Biosciences, Falcon, Franklin Lakes, NJ) with peptone-yeast extract/

Adhesion Assay of MPS-treated AC to SHCL

For the MPS study, trimmed Balafilcon A was used as the test disk. Fifty (50) μL of AC trophozoite suspension (1 × 10^5cells/mL in ¼ 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 previously in the above section. Seven lenses were used for each MPS solution.

Phase-contrast and Scanning Electron Microscopy (SEM) of Adherent AC

Fifty (50) μL of AC trophozoite suspension (5 × 10^5cells/mL in ¼ 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 twice the disk with ¼ 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), and 1% 100mM sodium cacodylate buffer (pH 7.4) for 1 hour at 4° C, then post-fixed with 1% OsO4 in the same buffer for 1 hour at 4° C. After standard graded dehydration with ethanol (50, 70, 80, 85, 90, 95, 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 (S-4800, Hitachi, Tokyo, Japan) at 5 kV.

Statistical Analysis

Statistical analysis of the number of adherent AC trophozoites and cysts to SHCLs was performed using analysis of variance with the Scheffe test or non-paired 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 five test SHCLs. The numbers of adherent AC trophozoites to Lotrafilcon B and Balafilcon A were 212±119 and 232±89 cells/disk. These numbers were significantly higher (ANOVA with Scheffe, $p<0.01$) in comparison to the numbers of adherent AC trophozoites to Asmofilcon A, Galyfilcon A and Senofilcon A, which were 22±6, 38±19 and 16±14 cells/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 (non-paired t-test, $p<0.0001$).

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/disk, respectively. These numbers were significantly (ANOVA with Scheffe, $p<0.01$) lower compared to the ¼ strength Ringer solution-treated disk (control), 143±60 cells/disk. Complete AminoMoist-treated AC showed relatively

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 to unworn lenses. However, the number of SE detected on worn conventional SCL, Etafilcon A, was significantly higher in comparison to 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 some studies on AC adherence to conventional SCL and SHCL *in vitro,* biofilm-coating and lens wear. Their results showed a significant higher number of AC adhered to SHCL, Lotrafilcon A and Balafilcon A, as compared to conventional SCL, Etafilcon A. However, there were no differences in AC number between unworn and worn Lotrafilcon A. In addition, no PA biofilm-coating was found. Our results showed a direct correlation between contact lens brands and AC trophozoites adhesion. On all SHCLs tested, AC-trophozoites adhesion was high with Lotrafilcon B and Balafilcon A. This result is consistent with reports showed that the attachment of AC to contact lenses is influenced by various parameters, including lens material properties, ionicity, water contents, surface hydrophobicity, protein-adsorption and lipid-adsorption properties.[22] Recently, Beattie et al.[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; however, it is very

high adherence, 64±39 cells/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 Mois (88±59 cells/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 to AC treated with Epica Cold, Aquamore or ReNu MultiPlus (Figure 3a, 3b, 3c, 3d). One-quarter (1/4) strength Ringer-treated trophozoites kept their normal shape (Figure 3a) compared to MPS-treated trophozoites, which become spherical (pre-cyst) after 24 hours. Both trophozoite and pre-cyst were observed at the same time in AC treated with OptiFree Plus, Complete AminoMoist, Complete DoubleMoist or C3 SoftOne Mois (Figure 3e, 3f, 3g, 3h).

Figure 4 shows representative scanning electron microscopic 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 Mois (Figure 4a, 4e, 4g, 4h) kept their trophozoite shape with expanding filopodia onto the lens surface. Epica Cold, Aquamore or ReNu MultiPlus treated AC (Figure 4b, 4c, 4d) changed into cystic shapes (pre-cyst form). Complete AminoMoist treated AC (Figure 4f) showed intermediate shape between trophozoite and cyst or deformed pre-cyst form.

DISCUSSION

Bacterial adhesion to conventional SCL and SHCL has been studied extensively using *Pseudomonas aeruginosa* (PA), *Serratia marcescens*, *Staphylococcus aureus* and

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 to four types of SCLs. FDA group I, ordinal poly-2-hydroxyethyl methacrylate lenses, showed the highest adhesion for both trophozoites and cysts. The results of Kilvington studies showed that trophozoites adhesion was 5 to 10 times higher than cysts adhesion. These results are similar with our results, which showed that trophozoites adhesion is 25 times higher than cysts adhesion. It is possible that adherence of trophozoite 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 hours treatment with two MPSs, MeniCare Soft (Epica Cold) and ReNu Multiplus.[17] Scanning electron microscopy analysis of AC morphology revealed that MeniCare Soft and ReNu MultiPlus-treated trophozoite changed into deformed cyst shapes, whereas Complete DoubleMoist and C3 SoftOne Mois-treated trophozoites kept their normal shape.[17] Four-hour exposure to Complete DoubleMoist and C3 SoftOne Mois had no effects on AC adhesion compared to control, 1/4 strength Ringer solution.[17] These results are similar to our present data showing AC trophozoites shape deformation after 24 hours of Epica Cold, Aquamore and ReNu Multiplus, whereas AC trophozoite treated with OptiFree Plus, Complete DoubleMoist and C3 SoftOne Mois kept their forms. Recently, Lonnen et al.[12] have compared disinfection efficacy of Epica Cold, Aquamore, OptiFree Plus and C3 SoftOne Mois using

Acanthamoeba castellanii (ATCC50370) and demonstrated that Epica Cold and Aquamore have significantly higher disinfecting efficacies against AC than OptiFree Plus and C3 SoftOne Mois. Beattie et al.[14] using number enumeration technique have also reported that ReNu MultiPlus had the highest efficacy compared to OptiFree Express, and Complete 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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Legends

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

Figure 2. Number of adherent *Acanthamoeba castellanii* treated with MPS to Balafilcon A.

AC trophozoites were mixed with seven 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

Figure 3. Phase-contrast microscopic images of adherent *Acanthamoeba castellanii* treated with MPS to Balafilcon A.

(Original magnification, × 200) (a) 1/4 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 Mois treatment for 24 hours.

AC trophozoites were mixed with seven MPSs and inoculated onto Balafilcon A and incubated for 24 hours.

Figure 4. Scanning electron microscopic images of adherent *Acanthamoeba castellanii*

treated with MPS to Balafilcon A.

(a)1/4 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 Mois treatment for 24 hours (Original magnification, × 3000).

AC trophozoites were mixed with seven MPSs and inoculated onto Balafilcon A and incubated for 24 hours.

Table1

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 indicates United States adopted names; SHCL, silicone hydrogel contact lens; PVP, poly vinyl pyrrolidone