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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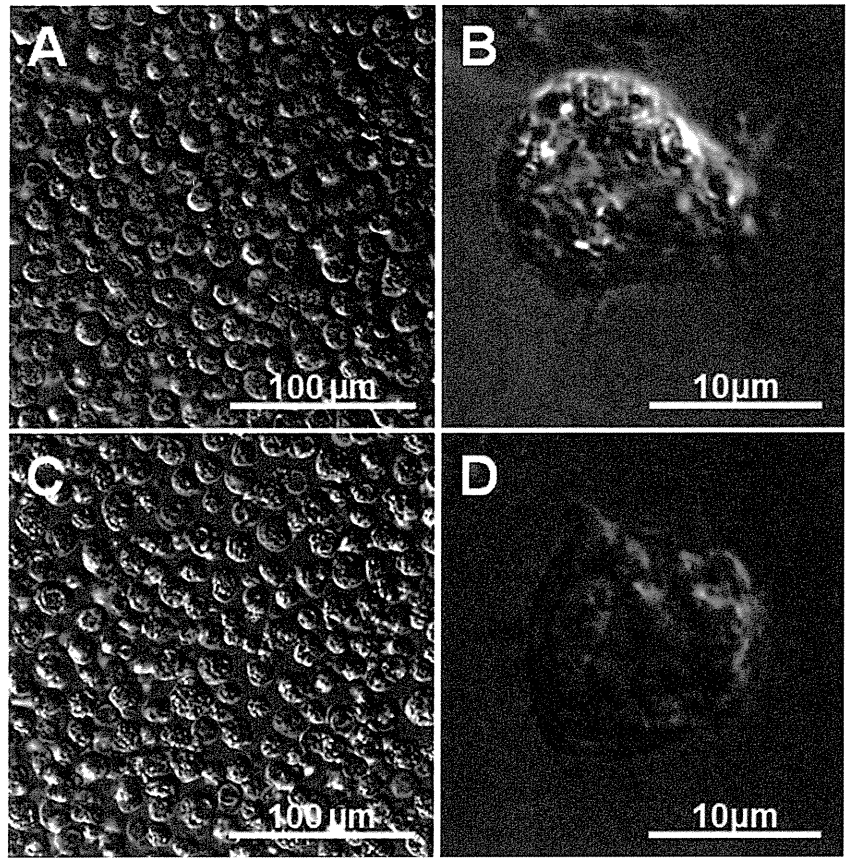

FIG 1 *Acanthamoeba castellanii* trophozoites stained with CTC. Merged fluorescence images of red-fluorescing CTC formazan and differential interference contrast images of trophozoites stained with CTC are shown. (A) CTC formazan accumulates within trophozoites, as shown by red fluorescence. (B) Trophozoite 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.

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

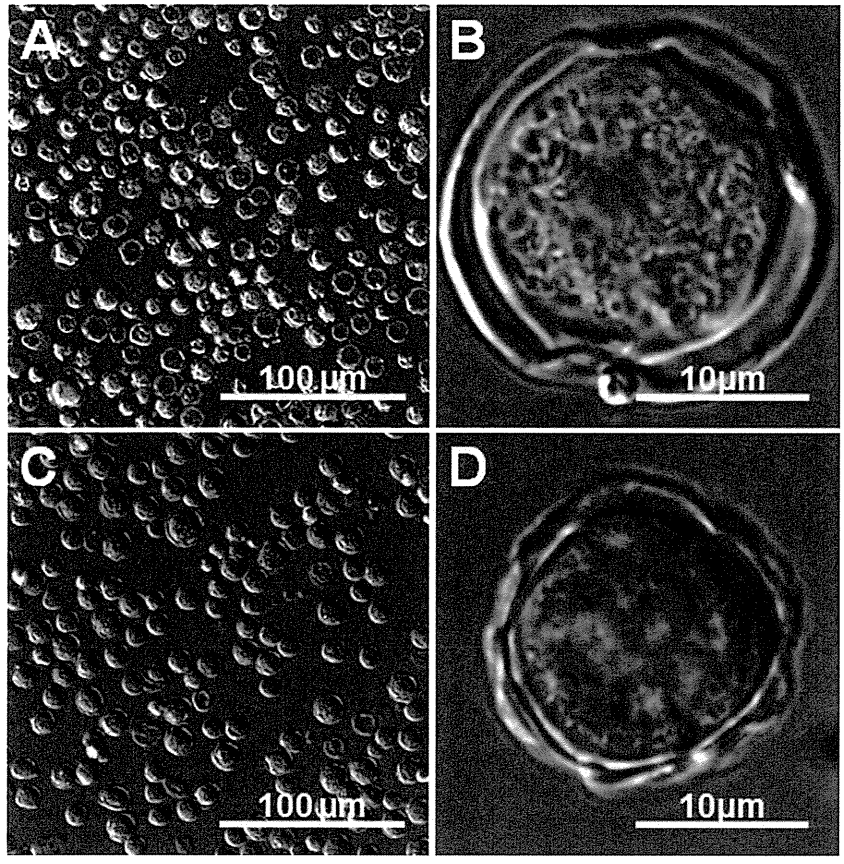

FIG 2 *Acanthamoeba castellanii* cysts stained with CTC. Merged fluorescence images of red-fluorescing CTC formazan and differential interference contrast images of cysts stained with CTC are shown. (A) CTC formazan accumulates within cysts, as shown by red fluorescence. (B) Cyst from panel A at higher 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

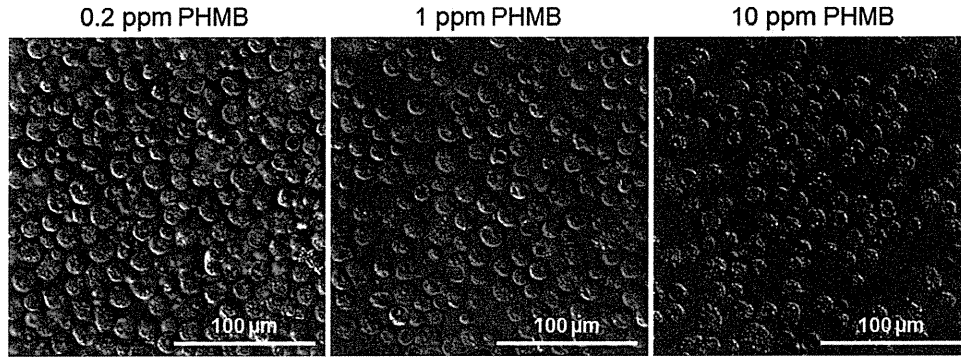

FIG 3 CTC staining of PHMB-treated *Acanthamoeba* trophozoites. Merged fluorescence images of red-fluorescing CTC formazan and differential interference contrast images of trophozoites stained with CTC after PHMB treatments are shown. CTC staining was performed on trophozoites treated with PHMB (0.2, 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,

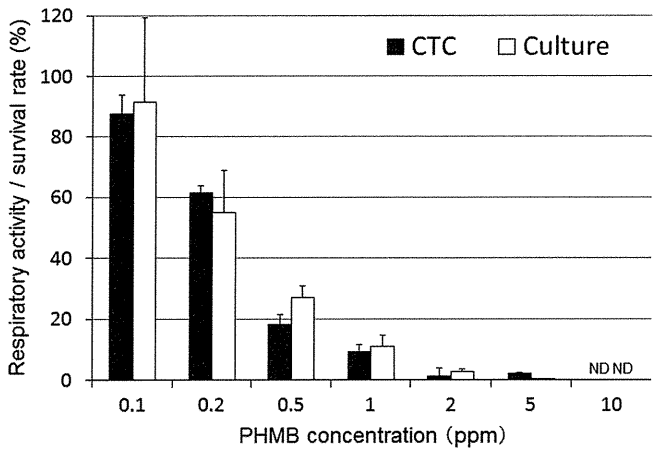

FIG 4 Respiratory activity and survival rate of *Acanthamoeba* trophozoites after PHMB treatment. The respiratory activity (CTC biocidal assay) was compared with the survival rate (culture-dependent biocidal assay) for trophozoites after treatment with PHMB (25°C, 4 h). Error bars represent the standard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

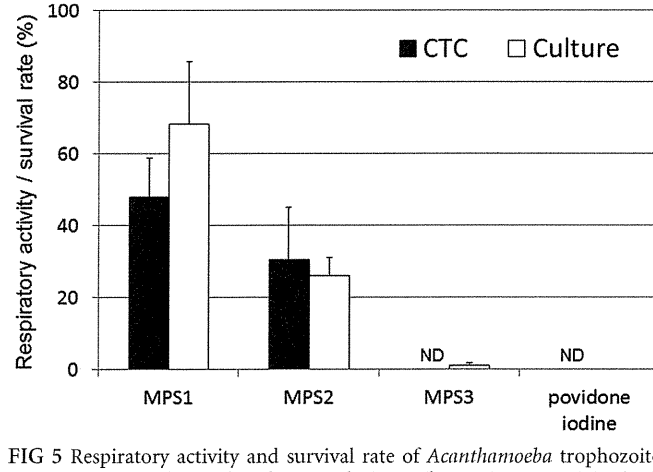

FIG 5 Respiratory activity and survival rate of *Acanthamoeba* trophozoites after treatment with SCL disinfectant solutions. The respiratory activity (CTC biocidal assay) was compared with the survival rate (culture-dependent biocidal assay) for trophozoites after treatment with SCL disinfectant solutions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

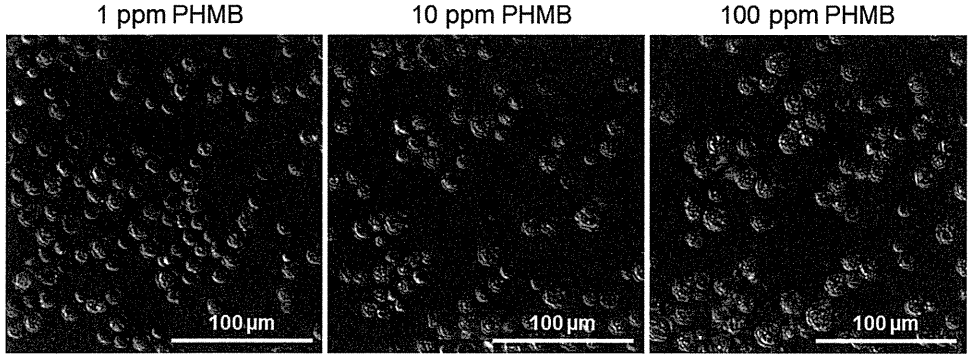

FIG 6 CTC staining of PHMB-treated *Acanthamoeba* cysts. Merged fluorescence images of red-fluorescing CTC formazan and differential interference contrast images of cysts stained with CTC after PHMB treatments are shown. CTC staining was performed on cysts treated with PHMB (1, 10, or 100 ppm) for 4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

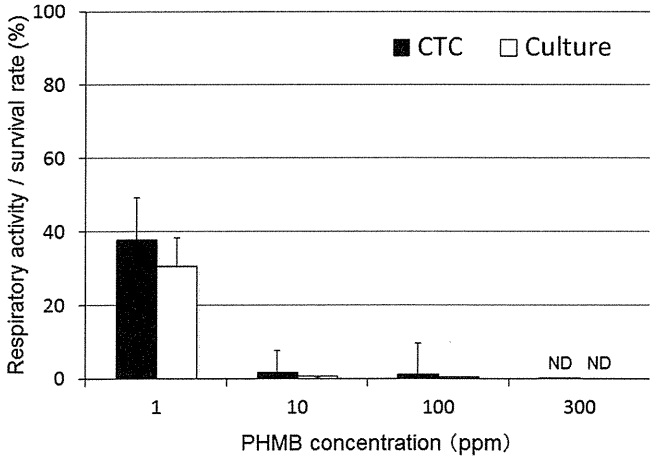

FIG 7 Respiratory activity and survival rate of *Acanthamoeba* cysts after PHMB treatment. The respiratory activity (CTC biocidal assay) was compared with the survival rate (culture-dependent biocidal assay) for cysts after treatment with PHMB (25°C, 4 h). Error bars represent the standard errors of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

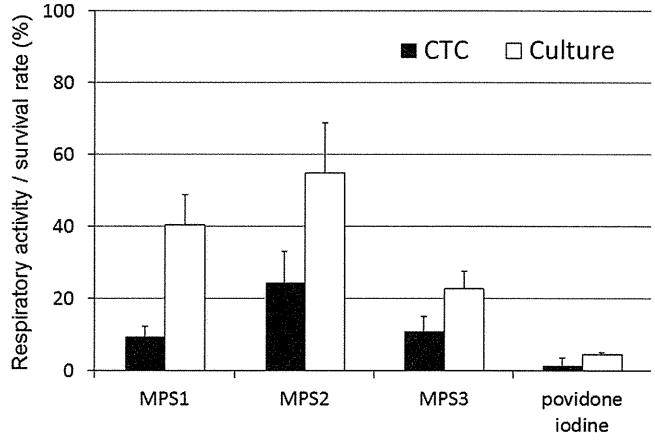

FIG 8 Respiratory activity and survival rate of *Acanthamoeba* cysts after treatment with SCL disinfectant solutions. The respiratory activity (CTC biocidal assay) was compared with the survival rate (culture-dependent biocidal assay) for cysts after treatment with SCL disinfectant solutions (25°C, 4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

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.

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

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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

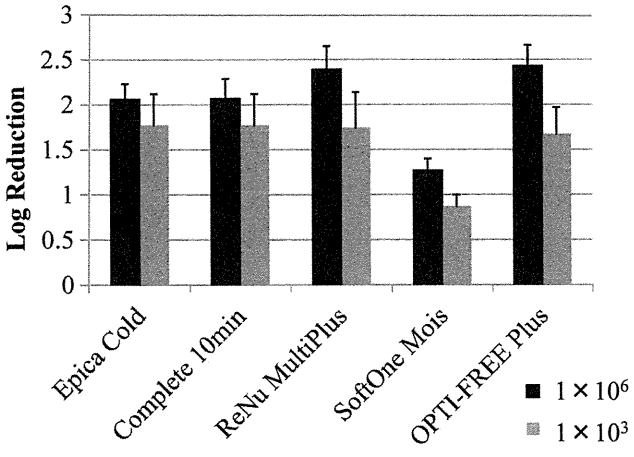

FIG. 1. The results of multipurpose solution (MPS) antimicrobial efficacy tests using ACUVUE2 lenses in contact lens cases. Final concentrations of the inoculants were 1×10^3 and 1×10^6 CFU per lens.

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

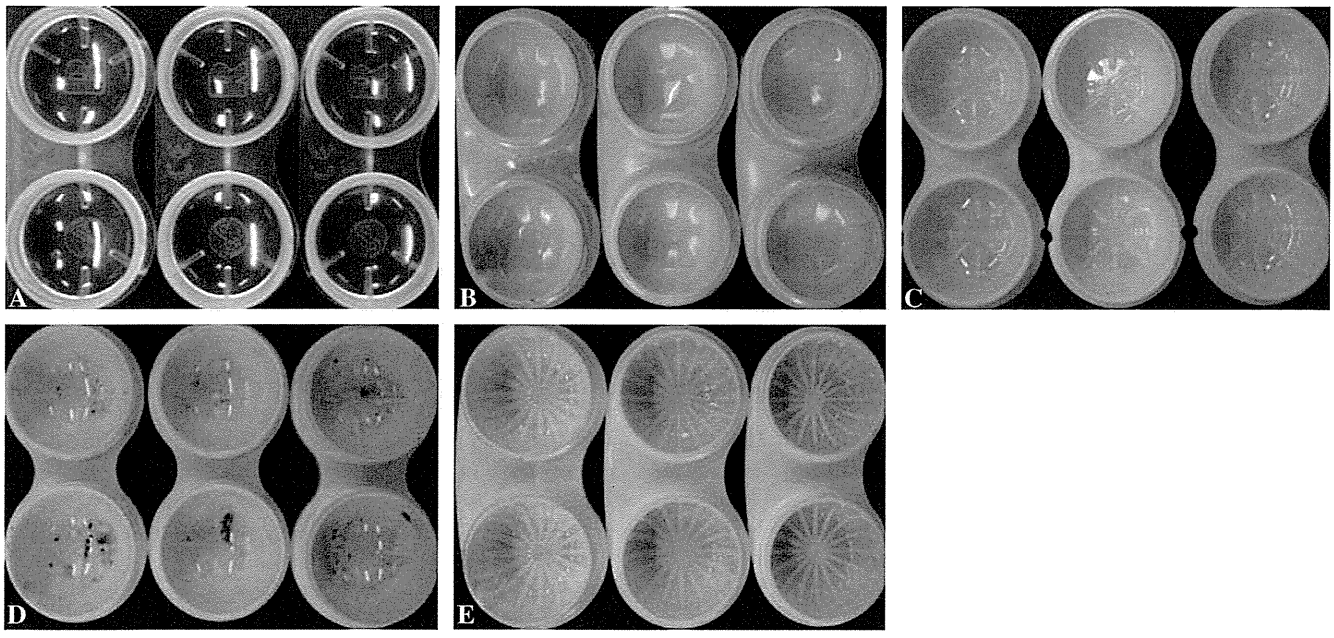

FIG. 2. View of each brand of lens storage case after 3 treatment cycles with each MPS. (A) Epica Cold; (B) Complete 10 min; (C) ReNu MultiPlus; (D) SoftOne Mois; (E) OPTI-FREE Plus.

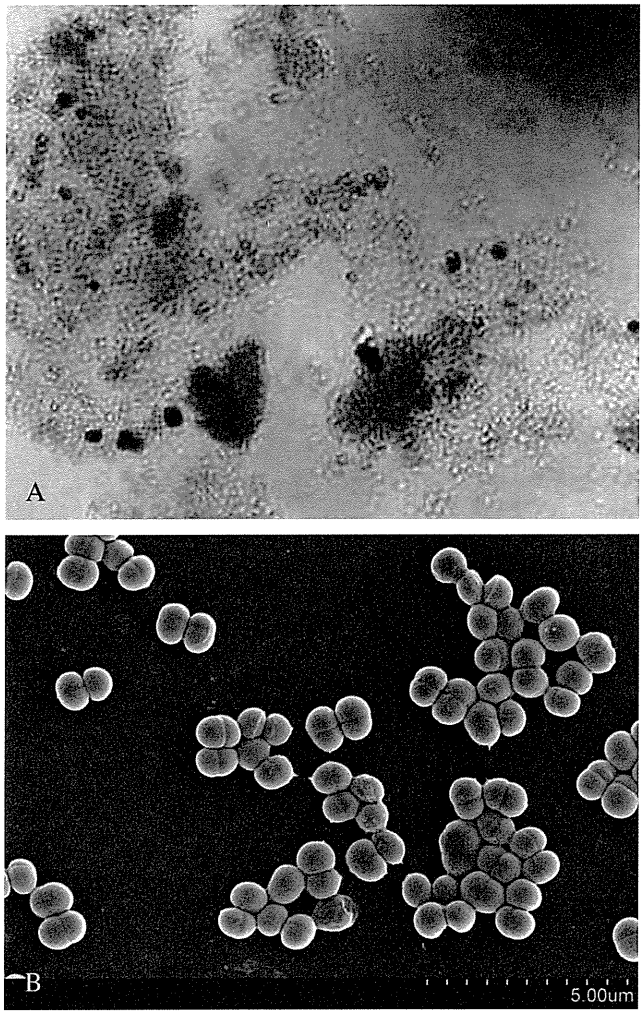

FIG. 3. Light and scanning electron microscopy (SEM) views of a lens storage case after 3 treatment cycles with SoftOne Mois. (A) Light micrograph (original magnification ×400); (B) SEM image (original magnification ×8,000).

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

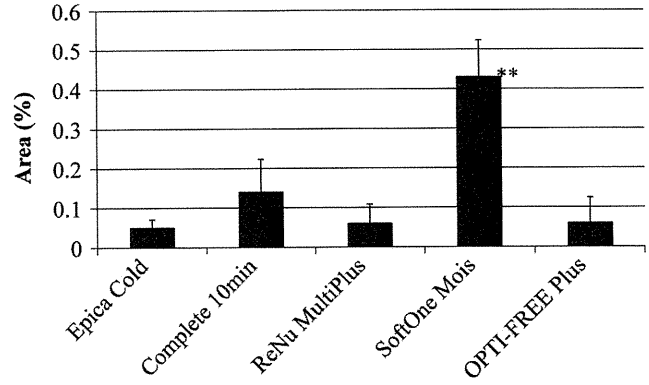

FIG. 4. The results of image analysis of safranin-staining area in a lens storage case. The percentage of safranin-staining area per each well of lens case was analyzed by image analysis. The error bars are SD obtained from six separate lens cases.

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

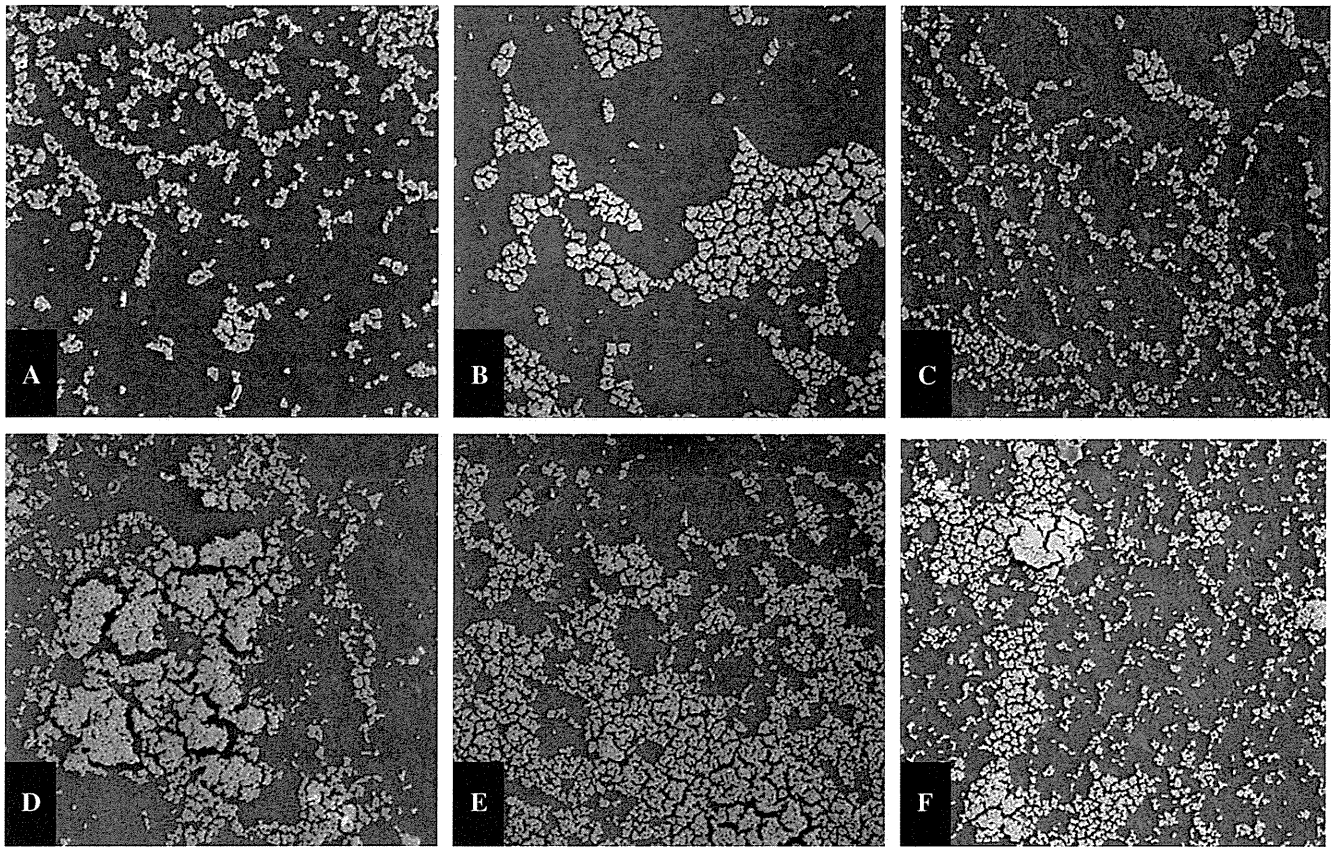

FIG. 5. Effects of MPS on *Staphylococcus epidermidis* (SE) biofilm evaluated by SEM observation (original magnification ×600). (A) EPica Cold; (B) Complete 10 min; (C) ReNu MultiPlus; (D) SoftOne Mois; (E) OPTI-FREE Plus; (F) control (saline).

note that disinfections at 1×10^3 and 1×10^6 CFU resulted in almost the same log reduction in all MPSs as shown in Figure 1. These results suggested that no shortage of preservatives occurred at the 1×10^6 CFU level. Higher inoculums at 1×10^7 or 1×10^8 CFU might cause shortage of preservatives resulting in smaller log reduction. Of the MPSs tested, Epica Cold and ReNu MultiPlus showed relatively high capacities to disinfect CL cases and breakdown SE biofilm compared with the other three MPSs. It was reported that antimicrobial efficacy of polyhexamethylene biguanide (PHMB) reduced when formulated in combination with a phosphate buffer system compared with when formulated in a borate buffer system.[22,23] Low efficacies of Complete 10 min and SoftOne Mois may be associated with combination of PHMB and phosphate buffer in these MPSs; however, the reason SoftOne Mois showed weaker efficacy than Complete 10 min was unknown. Other ingredients, such as surfactants, might be involved in the antimicrobial efficacies.

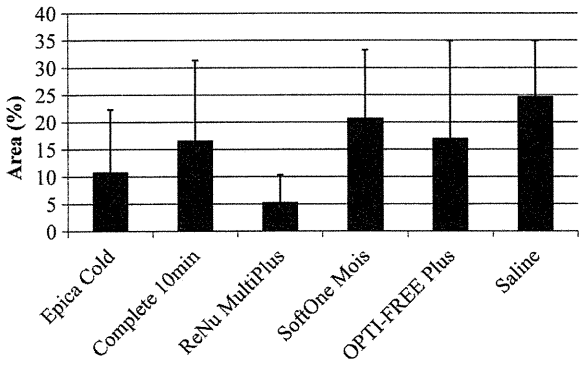

FIG. 6. The results of image analysis of biofilm-firmed area in a lens storage case. The percentage of SE biofilm-covered area in each SEM image (~30,500 μm^2) was analyzed by image analysis. The error bars are SD obtained from four separate lens cases.

In a previous study[24] conducted with SCL, we showed that Epica Cold and ReNu MultiPlus were the most effective against CA, whereas OPTI-FREE Pluslost its efficacy after 3 cycle treatments. In this study, we confirmed our previous results by testing SE with the same MPSs used. Epica Cold and ReNu MultiPlus were the most effective compare with OPTI-FREE Plus. On the other hand, Rosenthal et al.[25] showed that PHMB-based MPSs (ReNu MultiPlus, Complete Moisture Plus) decreased fungicidal efficacy more in comparison with POLYQUAD-based MPSs (OPTI-FREE Express, OPTI-FREE RepleniSH), which maintained their high fungicidal activity because of preservative uptake into/or adsorption onto the SCL. The discrepancy between the results of Rosenthal and those of ours may be because of experimental method differences. Rosenthal used a 1 hr to 7 days soaking time, whereas we used a treatment cycle of (lens soiling with serum—bacterial

inoculation—4 hrs of incubation with MPS). Furthermore, the discrepancy between the results of Rosenthal and those of ours may also result from differences between preservative in OPTI-FREE Express, POLYQUAD and Aldox, and that in OPTI-FREE Plus, POLYQUAD only.[26]

The findings presented here highlight the importance of compliant behaviors in preventing CL-related microbial keratitis. It further suggests the inefficacy of certain commercially available MPSs against SE biofilm that may increase the risk of SE-associated microbial keratitis under noncompliance behaviors, such as the lack of mechanical rubbing and subsequent rinsing. Greater efforts to educate patients regarding compliant lens care behavior are needed to reduce the incidence of CL-associated microbial keratitis.

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REFERENCES

1. Khor WB, Aung T, Saw SM, et al. An outbreak of *Fusarium* keratitis associated with contact lens wear in Singapore. *JAMA* 2006;295:2867–2873.
2. Saw SM, Ooi PL, Tan DT, et al. Risk factors for contact lens-related *Fusarium* keratitis: A case-control study in Singapore. *Arch Ophthalmol* 2007;125:611–617.
3. Rao SK, Lam PT, Li EY, et al. A case series of contact lens-associated *Fusarium* keratitis in Hong Kong. *Cornea* 2007;26:1205–1209.
4. Chang DC, Grant GB, O'Donnell K, et al. Multistate outbreak of *Fusarium* keratitis associated with use of a contact lens solution. *JAMA* 2006;296: 953–963.
5. Levy B, Heiler D, Norton S. Report on testing from an investigation of *Fusarium* keratitis in contact lens wearers. *Eye Contact Lens* 2006;32: 256–261.
6. Verani JR. Multistate outbreak of Acanthamoeba keratitis associated with use of a contact lens solution, presented in FDA Ophthalmic Devices Panel Meeting June 10, 2008. Available at: http://www.fda.gov/ohrms/dockets/ac/08/slides/2008-4363s1-03-VERANI-CDC.pdf.
7. Behlau I, Gilmore MS. Microbial biofilms in ophthalmology and infectious disease. *Arch Ophthalmol* 2008;126:1572–1581.
8. Suzuki T, Kawamura Y, Uno T, et al. Prevalence of *Staphylococcus epidermidis* strains with biofilm-forming ability in isolates from conjunctiva and facial skin. *Am J Ophthalmol* 2005;140:844–850.
9. Inoue N, Toshida H, Mamada N, et al. Contact lens-induced infectious keratitis in Japan. *Eye Contact Lens* 2007;33:65–69.
10. Butcko V, McMahon TT, Joslin CE, et al. Microbial keratitis and the role of rub and rinsing. *Eye Contact Lens* 2007;33:421–423.
11. Sutton SVW, Proskin HM, Keister DA, et al. A critical evaluation of the multi-item microbial challenge test in ophthalmic disinfectant testing. *CLAO J* 1992;18:155–160.
12. Study group of national surveillance of infectious keratitis in Japan. National surveillance of infectious keratitis in Japan [in Japanese]. *Nihon Ganka Gakkai Zasshi* 2006;110:961–972.
13. Ziebuhr W, Heilmann C, Gotz F, et al. Detection of the intercellular adhesion gene cluster (ica) and phase variation in *Staphylococcus epidermidis* blood culture strains and mucosal isolates. *Infect Immun* 1997;65:890–896.
14. Wu YT, Zhu H, Harmis NY, et al. Profile and frequency of microbial contamination of contact lens cases. *Optom Vis Sci* 2010;87:152–158.
15. ISO14729 Ophthalmic optics—Contact lens products—microbial requirements and test methods for products and regimes for hygienic management of contact lenses. 2001.
16. McGrath D, Costanzo SP, Manchester RJ. Comparative antimicrobial activity of no-rub multipurpose lens care solutions in the presence of organic soil. *Eye Contact Lens* 2003;29:245–249.
17. Fessia SL, Griffin MJ. A method for assaying biofilm capacity on polyurethane-coated slides. *Perit Dial Int* 1991;11:144–146.
18. McLaughlin-Borlace L, Stapleton F, Matheson M, et al. Bacterial biofilm on contact lenses and lens storage cases in wearers with microbial keratitis. *J Appl Microbiol* 1998;84:827–838.
19. Garcia-Saenz MC, Arias-Puente A, Fresnadillo-Martinez MJ, et al. Adherence of two strains of *Staphylococcus epidermidis* to contact lenses. *Cornea* 2002;21:511–515.
20. Gabriel MM, Schultz CL, Wilson LA, et al. Effect of *Staphylococcus epidermidis* on hydrogel contact lens retention on the rabbit eye. *Curr Microbiol* 1996;32:176–178.
21. Wu YT, Zhu H, Willcox M, et al. Removal of biofilm from contact lens storage cases. *Invest Ophthalmol Vis Sic* 2010;51:6329–6333.
22. 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.
23. Lloyd D, Turner LA, Khunkitti W, et al. Encystation in Acanthamoeba castellanii: Development of biocide resistance. *J Eurkaryot Microbiol* 2001;48:11–16.
24. Uno T, Ohashi Y, Imayasu M, et al. Antimicrobial efficacy of multipurpose solutions under various conditions of noncompliance with recommended lens care procedures. *J Jpn CL Soc* 2009;51:36–41.
25. Rosenthal RA, Dassanayake NL, Schlitzer RL, et al. Biocide uptake in contact lenses and loss of fungicidal activity during storage of contact lenses. *Eye Contact Lens* 2006;32:262–266.
26. Codling CE, Maillard J, Russell AD. Aspects of the antimicrobial mechanisms of action of a polyquaternium and an amidamine. *J Antimicro Chemo* 2003;51:1153–1158.

Assessment of Real-Time Polymerase Chain Reaction Detection of *Acanthamoeba* and Prognosis Determinants of *Acanthamoeba* Keratitis

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Objective: To evaluate the diagnostic value of real-time polymerase chain reaction (PCR) for detecting *Acanthamoeba* in eyes diagnosed with *Acanthamoeba* keratitis (AK) by conventional tests. In addition, to determine the preoperative prognosis-determining factors in eyes with AK.

Design: Retrospective, cross-sectional study.

Participants: A total of 104 eyes of 103 patients who were diagnosed with AK or with bacterial or bacteria-associated keratitis (BK) by conventional tests.

Methods: Twenty-nine eyes with AK and 75 eyes with BK were evaluated for *Acanthamoeba* and bacterial DNA by real-time PCR. The *Acanthamoeba* copy numbers, bacterial load, and clinical parameters in the patients with AK were assessed for those significantly associated with poor outcome, that is, final visual acuity of <20/50 or requiring keratoplasty, by logistic regression analysis.

Main Outcome Measures: Acanthamoeba DNA copy number, bacterial DNA copy number, and odds ratio (OR) for poor prognosis.

Results: The detection of amoebic DNA was 50 times more sensitive by real-time PCR than by conventional cyst counting. The *Acanthamoeba* copy numbers at the first visit (mean: $4.7\times10^5\pm3.2\times10^5$ copies) were significantly correlated with the AK stage, and both were significant risk factors for a poor outcome. The *Acanthamoeba* DNA copy numbers at the first visit and AK stage had a significantly high risk for poor outcome (OR of *Acanthamoeba* DNA copy per logarithm of copy numbers: 3.48, 95% confidence interval [CI], 1.04–111.63, $P<0.05$; OR of AK stage: 2.8 per stage increase, 95% CI, 1.07–7.30, $P<0.05$, after adjustment of age). In the AK cases with poor outcome, the amoebic DNA was not reduced by more than 90% after 1 month of treatment. The weak amoebic reduction was significantly associated with advanced AK stages or previous use of steroids. Bacterial 16S rDNA was detected in 53.6% of the eyes with AK, but it was not associated with any risk for refractoriness.

Conclusions: Real-time PCR was effective in detecting and managing AK. The *Acanthamoeba* copy number and AK stage at the first visit were significantly associated with poor outcome.

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Acanthamoeba keratitis (AK) is a destructive disease process with significant visual morbidity, and prompt diagnosis is important for good visual outcome. However, the available *Acanthamoeba* tests are generally not sensitive enough for precise diagnosis. At present, staining corneal smears with Calcofluor and Fungiflora Y is probably the most effective method of diagnosing AK.[1] However, false negatives can occur, which cause a delay of treatment leading to poor visual outcomes.

Another difficulty with AK is in evaluating the effectiveness of a treatment protocol because of the absence of reliable and quantitative methods of determining the *Acanthamoeba* copy numbers. In refractory cases, the immunologic responses can worsen even after weeks of intensive treatment, and dense infiltrations can then develop in the lesion. Under these conditions, qualitative tests (e.g., culturing and smear staining) do not provide sufficient information on whether the treatment has reduced the *Acanthamoeba* copy numbers. Thus, tests that are more sensitive and provide quantitative values of the *Acanthamoeba* copy numbers will be helpful.

Acanthamoeba is innocuous unless embedded in a diseased cornea or central nervous system. In addition, *Acanthamoeba* can be an opportunistic pathogen in immune-compromised hosts.[2] Free-living *Acanthamoeba* ingest mainly bacteria, and AK can develop in eyes with bacterial keratitis and bacteria-associated keratitis (BK).

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There is also a possibility that AK can develop in eyes with advanced BK, but because of the lack of sensitive and quantitative tests for *Acanthamoeba*, little information is available on whether such a cause is possible. To evaluate such cases, it would be valuable to determine the level of the *Acanthamoeba* copy numbers relative to the bacterial load in the lesion.

Polymerase chain reaction (PCR) measurements are known to have high sensitivity in detecting amoebic DNA.[3–6] Real-time PCR has high sensitivity and the ability to obtain quantitative values of the degree of AK.

Thus, this study determines the sensitivity and specificity of real-time PCR in detecting *Acanthamoeba* DNA in eyes with AK and with BK. In addition, we determined whether the copy numbers of *Acanthamoeba* were correlated with the bacterial load in eyes diagnosed with AK and BK by standard clinical tests. We also used logistic analysis to determine the clinical parameters that were significantly associated with poor outcomes. We shall show that the stage of the AK and the *Acanthamoeba* copy numbers at the initial examination are significantly associated with poor visual outcomes.

Materials and Methods

Diagnosis of *Acanthamoeba* Keratitis and Treatment

A total of 104 eyes of 103 patients with suspected infectious keratitis including AK and BK were studied between January 2006 and December 2010. Of these 103 patients, 49 were men and 54 were women, with a mean age of 48.2±2.2 years. Twenty-nine eyes of 28 patients were diagnosed with AK. Eleven were men and 17 were women, with a mean age of 26.4±1.7 years. Twenty-seven of the patients with AK (96.4%) were contact lens wearers.

Seventeen healthy subjects (9 male and 8 female) with a mean age of 36.4±1.4 years, who were not contact lens wearers, were enrolled for examination of their conjunctival scraping by real-time PCR as normal controls.

The diagnosis of AK was based on a modification of a described method.[4,5] A definitive diagnosis of AK was based on the clinical characteristics and identification of one or more of the following findings in the laboratory tests:[1–4] (1) identification of trophozoites or cysts in corneal scrapings stained with Fungiflora Y,[1] (2) positive *Acanthamoeba* cultures, (3) pathologic identification of *Acanthamoeba* cysts on keratoplasty specimens, and (4) identification of *Acanthamoeba* genome from corneal scrapings by PCR.

To collect tissues for staining or culture, the lesions were scraped or ablated to obtain a sufficient amount of tissue to maximize the amount of *Acanthamoeba* trophozoites or cysts. After this, the corneal bed was swiped, and the swab was processed for *Acanthamoeba* DNA.

The stage of the AK was determined at the first visit and based on the clinical findings observed by slit-lamp biomicroscopy.[4] Briefly, AK was divided into 5 stages of disease severity: 1 = epitheliitis, 2 = epitheliitis with radial neuritis, 3 = anterior stromal disease, 4 = deep stromal keratitis, and 5 = ring infiltrate or extra corneal inflammation.

After a definitive diagnosis of AK, most of the patients were treated with hourly instillations of 0.2% fluconazole, 1% voriconazole, 0.02% polyhexamethylene biguanide, 0.02% chlorhexidine gluconate, and 200 mg of oral itraconazole. The corneas were debrided to reduce the *Acanthamoeba* load and facilitate drug penetration.

The diagnosis of BK was based on positive microbial identification in smear staining or culturing. Cases with positive microbiological results and responsive to appropriate antibiotics were defined as BK. Other cases that did not meet any of the criteria were classified as BK.

The study protocol was approved by the Tottori University Ethics Committee, and the procedures used conformed to the tenets of the Declaration of Helsinki. An informed consent was obtained from all of the participants after an explanation of the procedures to be used.

Real-Time Polymerase Chain Reaction

DNA was extracted from the scrapings of the corneal lesions with the QIAamp DNA mini kit (Qiagen, Hilden, Germany).[6] The 18S rDNA of *Acanthamoeba*, which distinguishes it from other amoeba genera (*Hartmannella, Naegleria, Balamuthia, Nuclearia,* and *Vahlkampfia*), was amplified using reported primers and probe sets.[3]

Forward: 5'-CGACCAGCGATTAGGAGACG-3'

Reverse: 5'-CCGACGCCAAGGACGAC-3'

TaqMan Probe: 5'-FAM-TGAATACAAAACACCACCATCG-GCGC-BHQ

Real-time PCR was performed and analyzed using the LightCycler (Roche, Basel, Switzerland) under the following conditions: 95°C for 15 minutes, followed by 50 cycles at 95°C for 0 seconds, and 60°C for 1 minute.

A standard curve was created using a dilution series with known amounts of genomic DNA from *Acanthamoeba castellanii* ATCC30010D. A detection of more than 1 copy was classified as *Acanthamoeba* DNA positive.

The total bacterial load was determined by real-time PCR using a broad-range (universal) probe and primers sets that detect the 16S rDNA from the domain *Bacteria*.[7]

Forward: 5'- TCCTACGGGAGGCAGCAGT-3'

Reverse: 5'- GGACTACCAGGGTATCTAATCCTGTT-3'

TaqMan Probe: 5'-FAM- CGTATTACCGCGGCTGCTG-GCAC-BHQ

The bacterial copy number (i.e., the total bacterial load) was calculated by a standard curve generated by using defined numbers of cloned templates.

Statistical Analyses

Data are presented as the mean ± standard error of the means. To evaluate the significance of the differences between groups, unpaired *t* tests or Mann–Whitney *U* tests were used. Spearman correlation analysis was used to determine the coefficients of correlation (ρ) between factors. Chi-square and Fisher exact tests were used to test the significance of the associations between the 2 kinds of classification. Multivariate logistic regression analysis was carried out to compute the odds ratios (ORs) and the 95% confidence intervals (CIs). In bilateral AK cases, the visually poorer eye was used for the statistical analyses. $P<0.05$ was considered significant.

Results

Sensitivity of Real-Time Polymerase Chain Reaction in Detecting *Acanthamoeba*

Although the detection of microbes by real-time PCR is known to be sensitive, the degree of sensitivity for *Acanthamoeba* has not

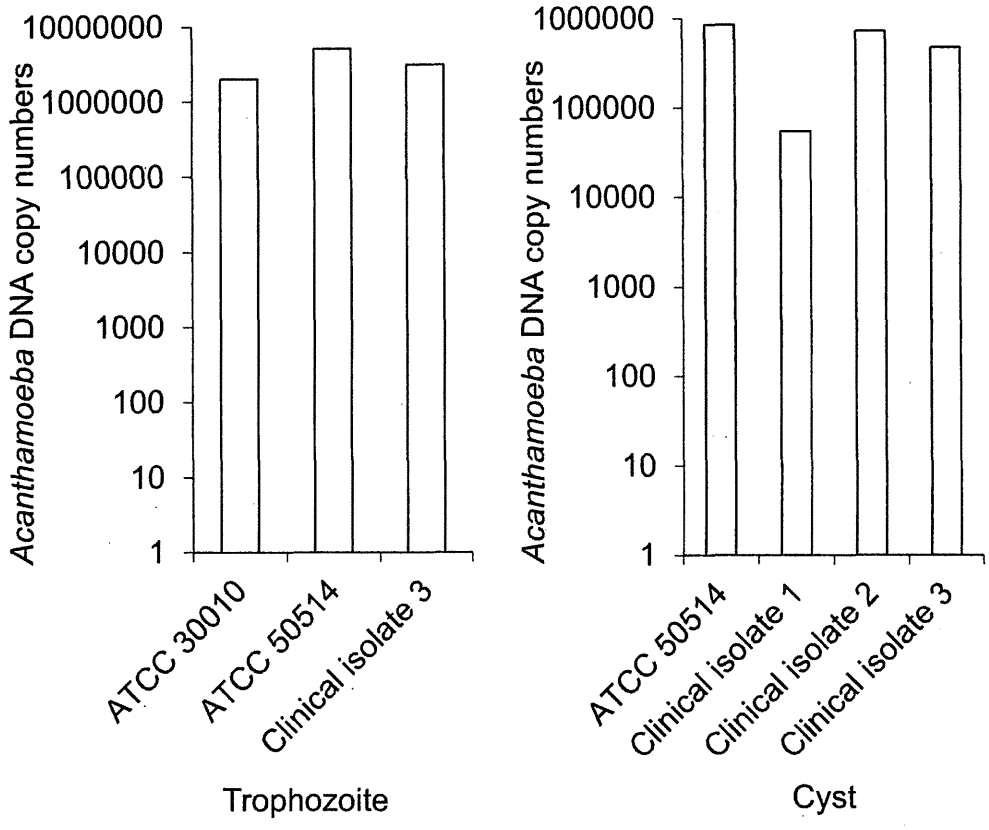

Figure 1. Evaluation of *Acanthamoeba* DNA detection in cultured *Acanthamoeba* cysts and trophozoites. Different strains of *Acanthamoebae* were cultured, and their cysts or trophozoites were diluted to 10 000 counts/ml. The extracted DNA was assessed for the *Acanthamoeba* DNA copy numbers. *Acanthamoeba* DNA detection by real-time polymerase chain reaction had better sensitivity for detecting both cysts and trophozoites. ATCC = American Type Culture Collection.

been determined. Generally, conventional microbiological tests rely on counting the number of amoebic bodies visually. Therefore, we first assessed how many copies of amoebic DNA can be detected for a known number of *Acanthamoeba* trophozoites or cysts (Fig 1). Clinical isolates and American Type Culture Collection strains were used as reference *Acanthamoeba,* and the trophozoites or cysts were diluted to 10 000 counts/ml in suspension.

Amoebic DNA was extracted from the suspension and assessed for the copy numbers using real-time PCR. Our results showed that the copy number detected by real-time PCR had approximately 300 times more sensitivity than visually counting trophozoites and 50 times more sensitivity than counting cysts (Fig 1).

To evaluate the specificity of *Acanthamoeba* real-time PCR in normal eyes, we also examined conjunctival scraping from 17 normal healthy subjects who were not contact lens wearers. No *Acanthamoeba* DNA (<1 copy) was detected in any of the scrapings.

Diagnostic Value of *Acanthamoeba* Real-Time Polymerase Chain Reaction

Acanthamoeba DNA was detected in 25 of the 29 AK eyes (86.2 %) by real-time PCR. The mean *Acanthamoeba* copy number was $4.7\times10^5\pm3.2\times10^5$ copies. The rate of detecting *Acanthamoeba* by smear staining with Fungiflora Y, which we have reported to be sensitive for detecting *Acanthamoeba,* was examined.[1] Among the 29 AK eyes, 26 underwent a Fungiflora test on diagnosis, and 22 eye samples were cultured. With Fungiflora Y staining, *Acanthamoeba* cysts were detected in 20 of 26 eyes (76.9%). In contrast, cultures of corneal specimens were positive in 12 eyes of 22 AK eyes (54.5%).

Acanthamoeba keratitis is generally associated with bacterial infection, but the specificity of identifying *Acanthamoeba* by real-time PCR has not been thoroughly evaluated in corneas diagnosed with BK. Therefore, we determined whether *Acanthamoeba* can be detected in BK cases using real-time PCR and Fungiflora Y staining. In 75 BK cases, none of the eyes was positive for *Acanthamoeba* DNA. Thus, the specificity of misdiagnosing AK by real-time PCR in eyes with BK was 100%.

When Fungiflora Y staining was assessed for specificity using 39 cases of BK, *Acanthamoeba* cyst-like staining was detected in 1 of 39 of the eyes with BK. This positive case was a contact lens user with *Pseudomonas aeruginosa* keratitis. Thus, the specificity of Fungiflora staining for diagnosing AK and not BK was 97.5%.

Evaluation of Bacterial Load by Real-Time Polymerase Chain Reaction

We next evaluated the bacterial load in the corneal lesions of eyes diagnosed as BK at their first visit or as a referral to Tottori University Hospital. For this, we used universal primers and real-time PCR for quantification of the bacterial DNA load. Bacterial

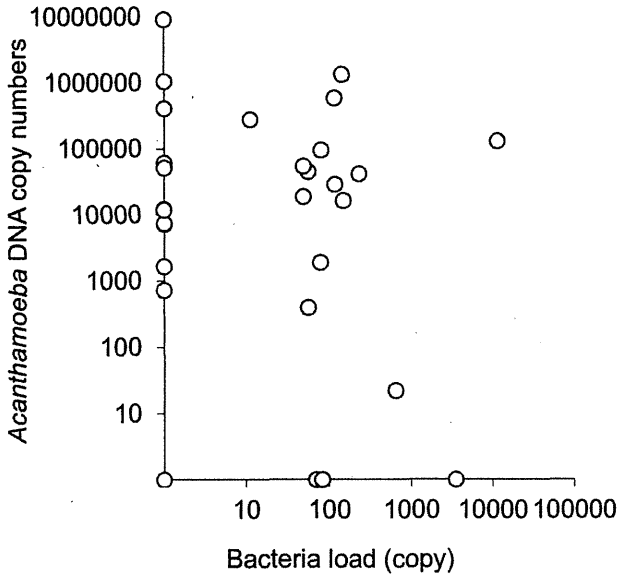

Figure 2. Evaluation of *Acanthamoeba* DNA copy number in *Acanthamoeba* keratitis determined by real-time polymerase chain reaction in relation to bacterial load. *Acanthamoeba* DNA copy number at first visit is not significantly correlated with the bacterial load in the lesion.

DNA was detected in 55 of 75 eyes (73.3%), and the mean bacterial DNA load was $8.8\times10^4\pm4.0\times10^4$ copies.

In the AK cases, 53.6% (15/28 eyes) were positive for bacterial DNA. The mean bacterial DNA copy number was $5.8\times10^2\pm4.0\times10^2$ copies. As expected, this was significantly lower than that in the BK eyes ($P<0.005$) by 10^2-fold.

To determine whether the development of AK was significantly associated with the bacterial load, we used Spearman correlation analysis to determine the relationship between the amoebic DNA copy number and the bacterial load in eyes with AK at the first visit. Amoebic DNA appeared inversely correlated with bacterial load (Fig 2); however, this was not statistically significant (Fig 2).

Correlation of *Acanthamoeba* DNA Copy Number and Stage of *Acanthamoeba* keratitis and Visual Acuity at First Visit

We next determined whether the stage of the AK was significantly associated with the copy number of *Acanthamoeba*. The *Acanthamoeba* copy number in the corneal specimens at the first visit was classified into 5 groups: 0 = not detected, 1 =≤1000, 2 =>1000 but ≤10 000, 3 =>10 000 but ≤100 000, and 4 =>100 000 copies. The relationship between the copy numbers of *Acanthamoeba* for each corneal specimen at the first visit to the stage of the AK was determined by Spearman correlation analysis. The stage of the AK was significantly correlated with the *Acanthamoeba* copy number ($\rho=0.53$, $P<0.05$; Fig 3A). In addition, the Acanthamoeba copy number was significantly correlated with the visual acuity in logarithm of the minimum angle of resolution units at the first visit ($\rho=0.37$, $P<0.05$; Fig 3B).

Next, the bacterial load was divided into 5 groups (0 = not detected, 1 =≤10, 2 =>10 but ≤100, 3 =>100 but ≤1000, 4 =>1000 but ≤10 000, and 5 =>10 000 copies). *Acanthamoeba* keratitis cases in the early stages were not significantly associated with the bacterial load (Fig 3C), and the correlation between the visual acuity and the bacterial load was not significant (Fig 3D). Thus, the *Acanthamoeba* copy number but not bacterial load was significantly associated with the disease severity and visual acuity before treatment.

We also determined whether the *Acanthamoeba* copy numbers at the first visit were significantly associated with the use of steroid or contact lens use. No significant associations were found (Mann–Whitney *U* test).

Parameters Associated with Poor Visual Outcome

We sought to determine factors that were significantly associated with poorer visual outcome in the AK cases after treatment of more than 2 months. A poor outcome was defined as a visual acuity <20/50 at the last visit or a requirement of keratoplasty. In AK cases with poor outcome, the *Acanthamoeba* copy numbers were 20 times higher than AK cases with good outcomes ($P<0.05$, Fig 4A). All of the cases with poor outcome had >10 000 copies of *Acanthamoeba*. When we evaluated the differences of the bacterial load between the AK cases with poor and good outcome, no significant difference was observed (Fig 4B). Thus, high *Acanthamoeba* DNA copy numbers at the first visit were associated with poor outcome.

We next evaluated the pretreatment parameters of the AK eyes that were significantly associated with poorer visual outcomes. By using logistic regression analysis, we calculated the risk of AK stage, *Acanthamoeba* DNA copy number at the first visit, bacterial load, previous use of steroids, and contact lens use (Table 1). We found that the *Acanthamoeba* DNA copy number and the stage of the AK at the first visit were the highest risk factors. The *Acanthamoeba* DNA copy numbers at the first visit had the highest risk for poor outcome (OR per category, 3.48; 95% CI, 1.04–111.63, $P<0.05$, after adjustment of age; Table 1). The AK stage had the second highest OR of 2.8 per stage increase (95% CI, 1.07–7.30, $P<0.05$, after adjustment of age). The previous use of steroids was not a significant risk (OR 8.84) for poor outcome ($P=0.07$). Other factors, including bacterial load and contact lens use, were not significant risk factors.

Parameters Associated with Unresponsive Reduction of *Acanthamoeba* Copy Numbers

Our findings indicate that the *Acanthamoeba* DNA copy number was significantly associated with the visual outcome. When we examined the *Acanthamoeba* copy number, all favorable outcome cases had a reduction in the copy number by >90% after 1 month of treatment (Fig 5A). In the cases with poor outcome, only 28.6% responded favorably to the treatment, and poor outcome was significantly associated with unresponsive reduction in the copy number.

To understand the cause of the outcome-related factors, an *Acanthamoeba* copy reduction was defined as a >90% reduction of *Acanthamoeba* copy numbers within 1 month of treatment. When the stage of the AK was compared between the responsive and unresponsive cases for *Acanthamoeba* copy reduction, the stage before the treatment of the unresponsive cases was significantly more advanced (Mann–Whitney *U* test, $P<0.01$, Fig 5B). The unresponsive *Acanthamoeba* copy reduction and previous steroid use were significantly associated ($P<0.05$, Fisher exact test). Five of the 23 unresponsive AK cases were previous steroid users, and the bacterial load in the unresponsive cases was not significantly associated with a decrease of *Acanthamoeba* copy numbers (unresponsive: 99±44 copies, responsive: 223±183 copies).

Finally, we determined which factors (e.g., the AK stage, amoebic DNA copy number, and bacterial load at the first visit) were significantly associated with an unresponsive DNA reduction. Logistic regression analysis was used to calculate the risks (Table 2). The AK stage was significantly associated with the DNA reduction with an OR of 8.00 per stage (95% CI, 1.06–

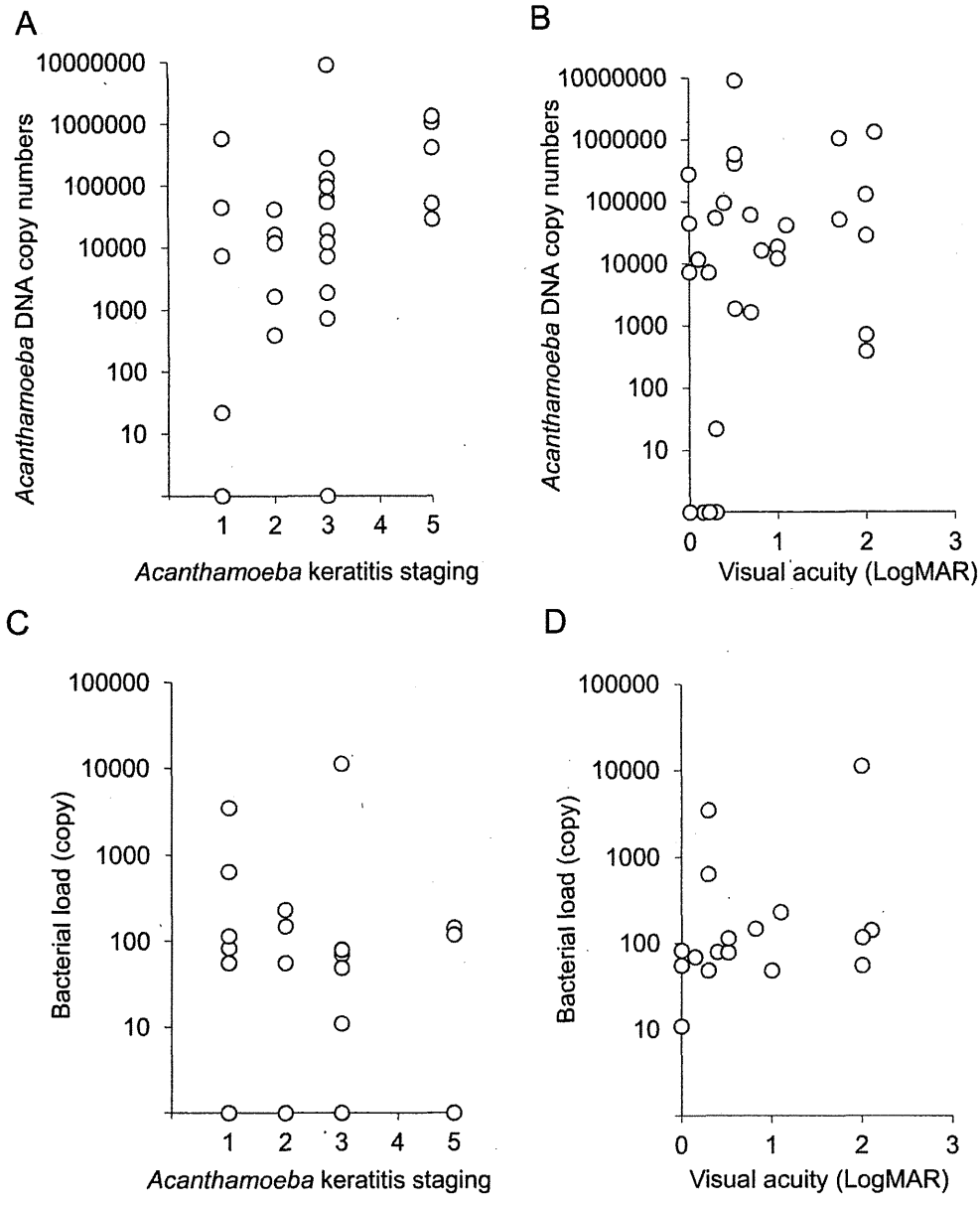

Figure 3. Significant correlation of *Acanthamoeba* DNA copy number with stage of *Acanthamoeba* keratitis (AK) and visual acuity. *Acanthamoeba* DNA copy number at first visit was significantly correlated with AK stage (ρ=0.53) **(A)** and logarithm of the minimum angle of resolution visual acuity (ρ=0.37) **(B)** by Spearman correlation analysis (P<0.05). Bacterial load in the lesion is not significantly correlated with the AK stage **(C)** or visual acuity **(D)**. logMAR = logarithm of the minimum angle of resolution.

58.82, P<0.05, after adjustment of age). The amoebic DNA copy number at the first visit also had a similar risk. The bacterial load at the first visit was not significantly associated with the DNA reduction.

Discussion

Acanthamoeba keratitis is a persistent infection and generally requires prolonged intensive treatment. However, our understanding of how to treat this disease remains undetermined. Presumably, the clinical presentation of AK (i.e., the stage of the AK) reflects the *Acanthamoeba* load, and the immunologic responses significantly affect its prognosis. Culturing, smear staining, and confocal microscopy are widely used for diagnosing AK. However, their qualitative nature and low sensitivity limit the amount of information that can be obtained to determine the cause of the AK. Thus, our initial aim was to determine how real-time PCR can be used for the diagnosis and management of AK.

Earlier studies with multivariate analysis of AK classified AK into 5 stages according to the corneal depth of the lesion and the severity of the corneal involvement as as-

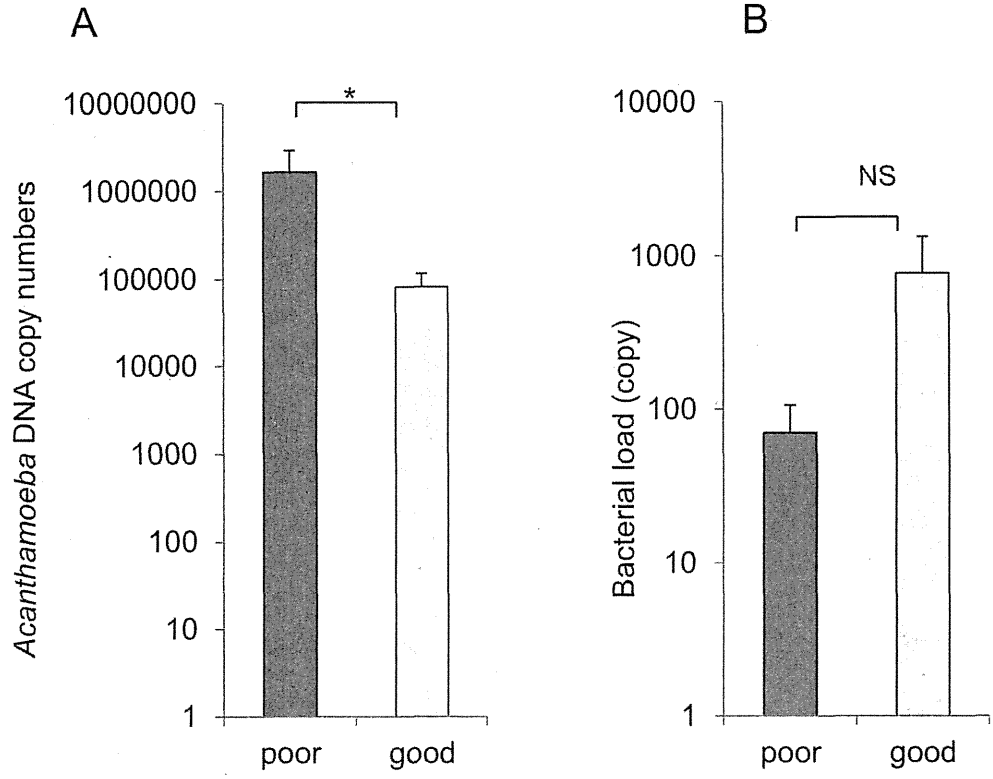

Figure 4. Elevation of *Acanthamoeba* DNA copy number in *Acanthamoeba* keratitis (AK) cases with poor outcomes. The AK cases with poor outcome, defined as visual acuity <20/50 at last visit or requiring keratoplasty, have a significantly higher number of *Acanthamoeba* DNA copies at the first visit compared with the AK eyes with good outcomes (A). Bacterial load at the first visit is not significantly different between them (B). *P<0.05. NS = not significant.

sessed by slit-lamp examinations.[4] The disease staging at presentation was predictive of worse outcomes and shown to help identify patients who might benefit from more aggressive therapy. Of note, advanced-stage AK with deep stromal involvement and ring infiltrates was associated with worse outcomes.[4,8,9]

Then, the important question arose on how much *Acanthamoeba* load is present at each stage. Our results showed that there was a strong and positive correlation of each stage with the *Acanthamoeba* DNA copy number, that is, more advanced stages of AK had higher *Acanthamoeba* DNA copy numbers. This explains why more advanced stages of AK were so refractory to treatment. Although the living amoebic bodies were fewer (Fig 1), this means that destroyed amoeba-derived DNA is abundant. Thus, the strong immunologic responses of the host to *Acanthamoeba*, manifested as ring infiltrate, are still not effective in eliminating the high *Acanthamoeba* copy numbers.

The most obvious advantage of real-time PCR is its high sensitivity.[3,10,11] Real-time PCR for *Acanthamoeba* genomic DNA will detect both live and destroyed amoeba. This was shown in our analysis of cultured *Acanthamoeba* cysts or trophozoites (Fig 1). Cultured *Acanthamoeba* samples generally are accompanied by fragments of the dead bodies and DNA. Real-time PCR attains its greater sensitivity in detecting

Table 1. Parameters Associated with Poor Outcome of *Acanthamoeba* Keratitis by Logistic Regression Analysis

	Odds Ratio					
	Lowest Category	*Second Category*	95% CI	*Highest Category*	95% CI	*P Value*
Acanthamoeba DNA copy number at the first visit	1.0	≤1000:3.48	1.04–111.63	>100 000:147.39	1.18–18 281.3	0.04*
AK stage	1.0	Stage 2:2.8	1.07–7.30	Stage 5:61.56	1.31–2838.69	0.04*
Bacterial load at the first visit	1.0	≤10:0.88	0.47–1.64	>10 000:0.53	0.02–11.86	0.69
Previous use of steroids	(-):1	8.84	0.852–91.68			0.07
Contact lens use	(-):1					0.996

AK = *Acanthamoeba* keratitis.
*P≤0.05.