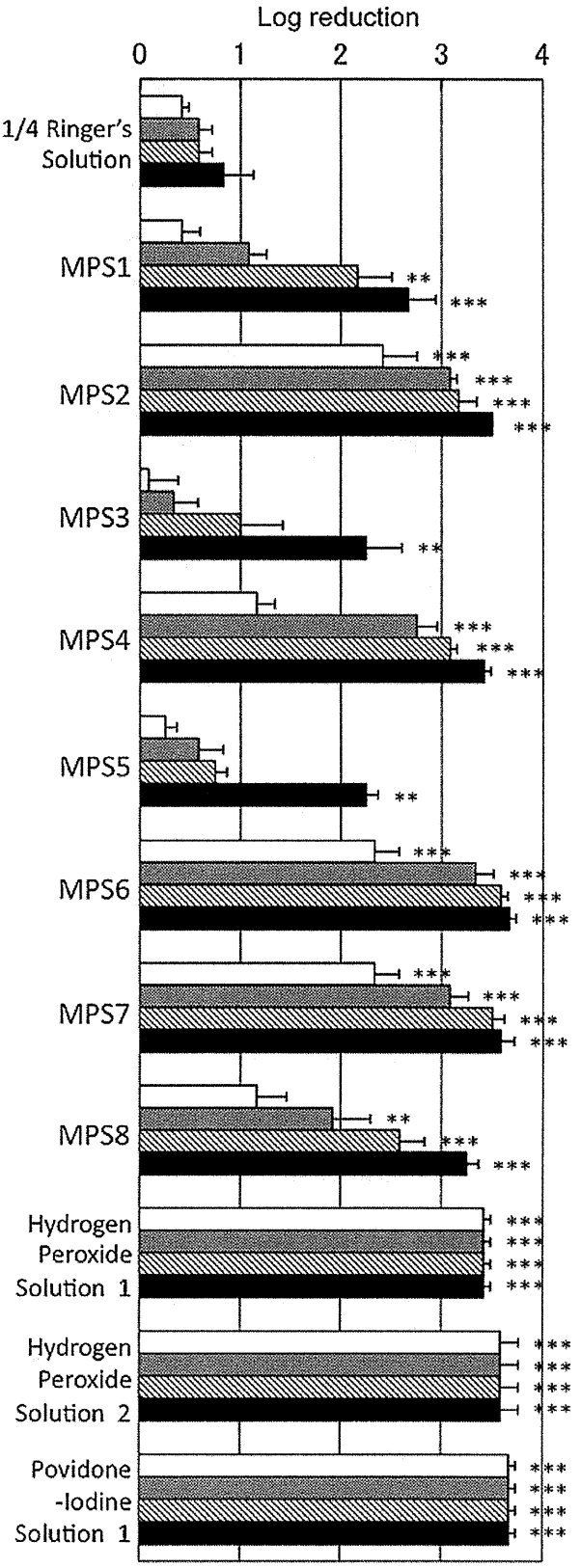


◀**Fig. 2** Efficacy of commercial soft contact lens disinfectant solutions against 1-week-old *Acanthamoeba* cysts (ATCC 50514). Eight types of multipurpose solution (MPS; $n = 4$), two types of hydrogen peroxide solution ($n = 3$), and one povidone–iodine solution ($n = 3$) were examined to determine their efficacy against 1-week-old *Acanthamoeba* cysts. 1/4 Ringer's solution was used as the control ($n = 7$). *White bar* 2-h treatment; *gray bar* 4-h treatment; *hatched bar* 8-h treatment; *black bar* 24-h treatment. The decrease in the number of surviving organisms in each solution was expressed logarithmically (log reduction value). The *error bars* indicate SEM. The log reduction value for each solution was compared with that for the control (*P value 0.01–0.05; **P value 0.001–0.01; ***P value <0.001)

iodine solution yielded more than a 3 log reduction in 1-week-old cysts, significantly greater than that produced by the control ($P < 0.001$) (Fig. 2).

Efficacy of soft contact lens disinfectant solutions against 2-week-old cysts

The MPS examined in this study were less effective against 2-week-old cysts than against trophozoites or 1-week-old cysts (Fig. 3). When the 2-week-old cysts were treated with any of the eight MPS for the manufacturer's recommended disinfection time (10 min–4 h), no statistically significant differences were observed between the log reduction values for the MPS and the control (1/4 Ringer's solution). Compared with all other MPS tested in this study, MPS 7 and 8 (PHMB 1.0 ppm) were more effective against 2-week-old cysts, producing a 1.4–1.8 log reduction after treatment for 24 h, significantly greater than that produced by the control ($P = 0.03$ and 0.004 for MPS 7 and 8, respectively).

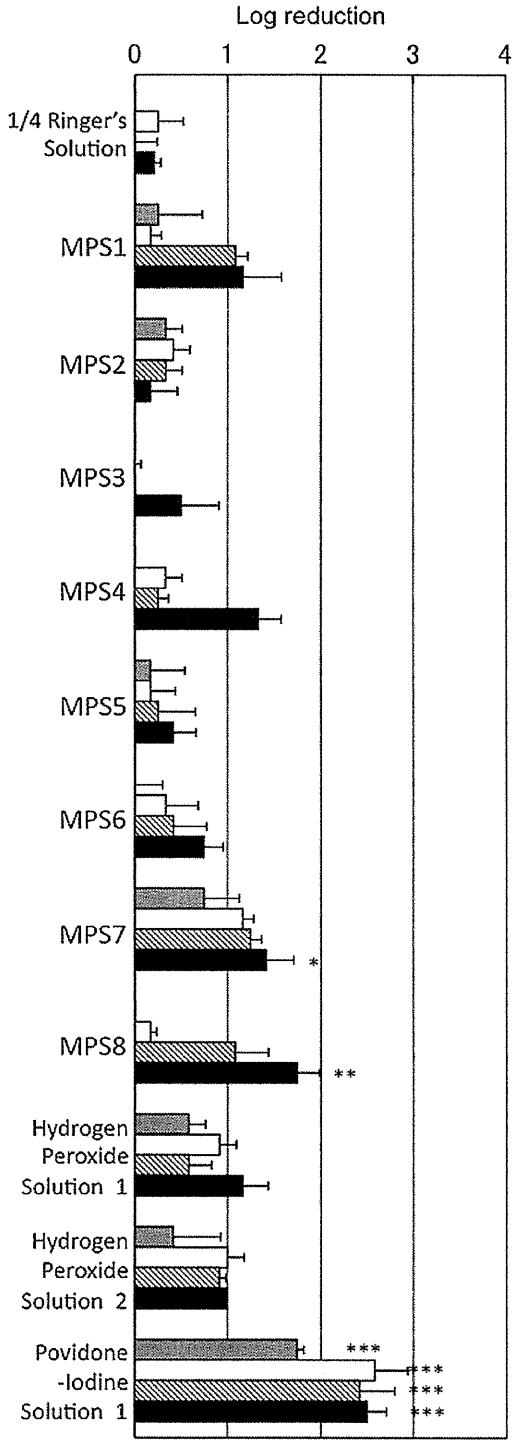
In contrast with the results for trophozoites and 1-week old cysts, the hydrogen peroxide solutions were ineffective against 2-week-old cysts (Fig. 3). When the 2-week old cysts were treated for 2, 4, 8, or 24 h with either hydrogen peroxide solution, no statistically significant differences were observed between the log reduction values for the hydrogen peroxide solutions and the control (1/4 Ringer's solution).

The povidone–iodine solution had greater disinfectant efficacy against the 2-week-old cysts than the MPS and the hydrogen peroxide solutions.

When the 2-week-old cysts were treated with povidone–iodine solution for 4 h, a 2.6 log reduction was achieved, significantly greater than that produced by the control ($P < 0.001$) (Fig. 3).

Verification of the log reduction method

Table 3 shows the efficacy of disinfectant solutions against *Acanthamoeba* trophozoites as measured by inoculating agar plates coated with *E. coli* with the treated samples. The results show that MPS 6 and povidone–iodine solution



◀ **Fig. 3** Efficacy of commercial soft contact lens disinfectant solutions against 2-week-old *Acanthamoeba* cysts (ATCC 50514). Eight types of multipurpose solution (MPS; $n = 3$), two types of hydrogen peroxide solution ($n = 3$), and one povidone–iodine solution ($n = 3$) were examined to determine their efficacy against 2-week-old *Acanthamoeba* cysts. 1/4 Ringer's solution was used as the control ($n = 6$). *White bar* 2-h treatment; *gray bar* 4-h treatment; *hatched bar* 8-h treatment; *black bar* 24-h treatment. The decrease in the number of surviving organisms in each solution was expressed logarithmically (log reduction value). The *error bars* indicate SEM. The log reduction value for each solution was compared with that for the control (*P value 0.01–0.05; **P value 0.001–0.01; ***P value <0.001)

were effective against *Acanthamoeba* trophozoites whereas MPS 1 and 5 had no observable effect. When tested against 2-week old *Acanthamoeba* cysts, MPS 1, 5 and 6 were ineffective, whereas povidone–iodine solution was effective. These results are consistent with those obtained using the log reduction method, and suggest that the log reduction method is a reliable means of evaluating disinfectant efficacy (Table 4).

Evaluation of disinfectant solution efficacy against *A. castellanii* (ATCC 50370)

Figure 4 shows the efficacy of selected disinfectant solutions against *A. castellanii* (ATCC 50370) as measured by the log reduction method. MPS 1 and 5 were not statistically different from the control (1/4 Ringer's solution), whereas MPS 6 and povidone–iodine solution had high biocidal activity against trophozoites; these solutions produced a 2.2–3.4 log reduction, significantly higher than that found for the control ($P < 0.001$). When 2-week-old cysts were treated with povidone–iodine solution, a 1.1 log reduction was achieved, significantly greater than that produced by the control ($P < 0.001$). However, no statistically significant differences were observed between the log reduction values for MPS 1, 5, or 6 and the control (1/4 Ringer's solution) (Fig. 4). These results are similar to the results obtained for *A. castellanii* (ATCC 50514), and suggest that the two strains respond similarly to the disinfectant solutions tested in this study.

Discussion

This study investigated the efficacy of commercially marketed SCL disinfectant solutions against *Acanthamoeba* trophozoites and cysts. MPS had widely variable effects on the organisms, despite the fact that all solutions except for MPS 5 use PHMB as the disinfecting agent. Most MPS currently marketed in Japan contain 1 ppm PHMB, which is within the range of the minimum trophozoite amoebicidal concentration for PHMB (0.87–1.3 ppm; minimum concentration required for complete destruction of trophozoites in 24–48 h), but lower than the established minimum cysticidal concentration (2.11–3 ppm; minimum concentration required to prevent excystment and trophozoite replication) [24–26]. This may explain why the MPS tested in this study were ineffective against 2-week-old cysts.

Table 3 Efficacy of commercial soft contact lens disinfectant solutions against *Acanthamoeba* trophozoites (ATCC 50514) tested by culturing samples on agar plates coated with *E. coli*

	Number of plates positive for *A. castellanii* (%)		
	Experiment 1	Experiment 2	Experiment 3
1/4 Ringer's solution	3/3 (100)	3/3 (100)	3/3 (100)
MPS 1	3/3 (100)	3/3 (100)	3/3 (100)
MPS 5	3/3 (100)	3/3 (100)	3/3 (100)
MPS 6	0/3 (0)	0/3 (0)	0/3 (0)
Povidone–Iodine Solution 1	0/3 (0)	0/3 (0)	0/3 (0)

Table 4 Efficacy of commercial soft contact lens disinfectant solutions against *Acanthamoeba* cysts (ATCC 50514) tested by culturing samples on agar plates coated with *E. coli*

	Number of plates positive for *A. castellanii* (%)		
	Experiment 1	Experiment 2	Experiment 3
1/4 Ringer's solution	3/3 (100)	3/3 (100)	3/3 (100)
MPS 1	3/3 (100)	3/3 (100)	3/3 (100)
MPS 5	3/3 (100)	3/3 (100)	3/3 (100)
MPS 6	3/3 (100)	3/3 (100)	3/3 (100)
Povidone–Iodine Solution 1	3/3 (100)	1/3 (33.3)	1/3 (33.3)

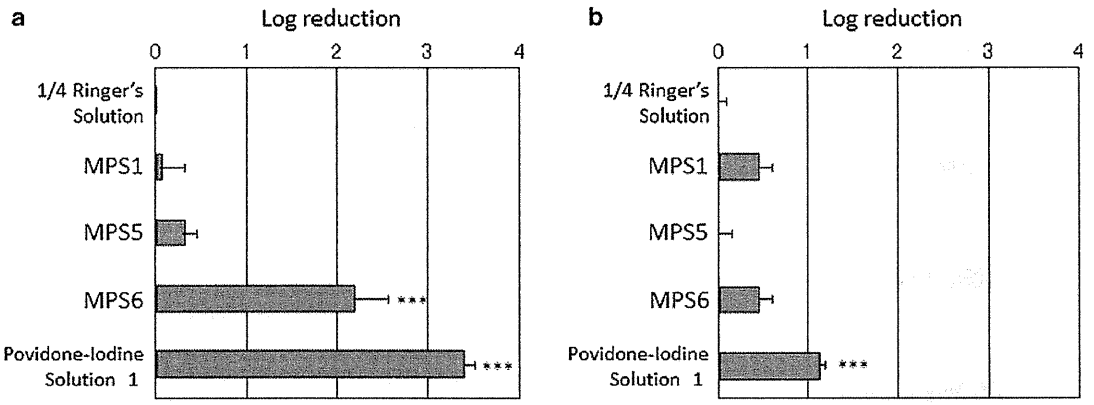


Fig. 4 Efficacy of commercial soft contact lens disinfectant solutions against *Acanthamoeba* trophozoites and cysts (ATCC 50370). Three types of multipurpose solution (MPS; $n = 4$) and one povidone–iodine solution ($n = 4$) were examined to determine their efficacy against *A. castellanii* ATCC 50370 trophozoites (**a**) and cysts (**b**). 1/4 Ringer's solution was used as the control ($n = 4$). The decrease in the number of surviving organisms in each solution was expressed logarithmically (log reduction value). The *error bars* indicate SEM. The log reduction value for each solution was compared with that for the control (*P value 0.01–0.05; **P value 0.001–0.01; ***P value <0.001)

With regard to MPS activity against trophozoites, our study found that many types of MPS are ineffective, although MPS 6 and MPS 7 had strong biocidal activity similar to that of hydrogen peroxide or povidone–iodine solutions (>3 log reduction after 4 h). Numerous previous studies suggest that the effect of PHMB may be altered by inactive ingredients included in the commercially marketed contact lens disinfectant solutions. Beattie et al. [10] evaluated the efficacy of six different types of MPS containing either PHMB or Polyquad against *Acanthamoeba*, and reported that even MPS which contained the same concentration of the disinfectant had different disinfectant efficacy. Similar phenomena were reported for the effects of different formulations of disinfectants against bacteria and fungi [27]. These differences were attributed to inactive ingredients in MPS, for example buffering agents, stabilizing agents, isotonizing agents and surfactants, which may interfere with the mechanism of PHMB. PHMB contains biguanide, a cation which enables it to adhere to the negatively charged surface of microorganisms. Upon adhering to phosphate-containing compounds, PHMB induces changes in cell membrane permeability leading to potassium efflux and eventual loss of membrane function and cell death [23]. Therefore, negatively charged

ingredients are particularly likely to interact electrostatically with PHMB and interfere with its ability to adhere to cell membranes and compromise their integrity [27].

Several types of inactive ingredient found in the products tested in this study may affect their efficacy. Buffering agents ensure that the pH of the MPS is similar to that of tears, and are necessary to minimize discomfort during lens wear. Most MPS contain phosphate, borate, or citrate buffers (Table 2). Of these, citrate has been found to attenuate the effect of PHMB against bacteria and fungi [28], possibly because citrate contains a negatively charged carboxyl group which interacts electrostatically with positively charged PHMB. Most stabilizing agents (EDTA and polylysine) and isotonizing agents (NaCl, KCl, amino acids) used in MPS are also ionic compounds (Table 2). Some of these ingredients, for example EDTA, have intrinsic disinfectant effects, but these ingredients also have the capacity to interact electrostatically with positively charged PHMB. One study found that EDTA can actually attenuate the effect of PHMB on *Acanthamoeba* [30]; it is, therefore, still unclear whether it is beneficial to include EDTA in MPS.

MPS also contain several types of surfactant, which are included to remove protein residues from lenses and to improve comfort during lens wear. Most surfactants found in MPS are nonionic compounds (poloxamer, poloxamine, propylene glycol), and are not likely to directly affect the activity of PHMB. However, these ingredients are reported to contribute to the survival of *Acanthamoeba*, because of their tendency to protect microorganisms by aiding biofilm formation and inducing amoebal encystment [31, 32]. Nevertheless, much about the individual and collective effects of these compounds on the disinfectant efficacy of PHMB remains unclear, and further studies are necessary to clarify these matters.

The hydrogen peroxide and povidone–iodine solutions used in this study were effective against *Acanthamoeba* trophozoites, but less effective against 2-week-old cysts, especially hydrogen peroxide. All of these solutions were the one-step type, meaning that neutralization was initiated concurrently with the start of disinfection. Therefore, during the 4 h allowed for disinfection, it is possible that the neutralization tablets or platinum disks may have neutralized the active ingredients (hydrogen peroxide or povidone–iodine) such that the concentration of active disinfectant prematurely fell below that needed for adequate disinfection. This theory is supported by the fact that both the hydrogen peroxide and povidone–iodine solutions caused a decrease in the number of surviving organisms during the first 4 h of treatment, but no further significant decrease in surviving organisms was observed during 20 subsequent hours. In addition, previous studies show that two-step hydrogen peroxide solutions, which enable neutralization to be carried out after disinfection, are more effective than one-step solutions against *Acanthamoeba* [33, 34]. If adequate disinfection is the top priority, two-step solutions may be ideal. However, as these solutions are more complicated, and are associated with the risk of forgetting to neutralize the solution, further discussion is necessary to determine how best to resolve this dilemma. One possible solution may be to improve the efficacy of one-step solutions by making adjustments to the formulation that delay the onset of neutralization.

Acanthamoeba cysts are highly resistant to many forms of environmental stress, including desiccation, ultraviolet light, and extreme cold or heat, and can, therefore, survive in a variety of different environments [29, 35]. Encystment also enables organisms to gain increased resistance to chemical stresses such as disinfectants [10, 14]. MPS and hydrogen peroxide solutions tested in this study were less effective against 2-week-old cysts than against trophozoites. All the MPS tested failed to significantly reduce numbers of 2-week-old cysts compared with the control (1/4 Ringer's solution) within the manufacturer's recommended disinfection time (4 h). In addition, consistent with previous reports [14, 17, 35], this study found that both types of hydrogen peroxide solution were also relatively ineffective against 2-week-old cysts. These results suggest that MPS and hydrogen peroxide solutions do not provide adequate protection against mature cysts. In contrast, treatment with the povidone–iodine solution led to an approximately 2.5 log reduction in 2-week-old cysts within the manufacturer's recommended disinfection time (4 h), efficacy superior to that of all the other solutions tested.

When the effects of disinfectant solutions against 1 and 2-week-old cysts were compared in this study, 2-week-old cysts were found to be highly resistant to all solutions, whereas 1-week-old cysts were either equally or slightly more susceptible to the solutions than trophozoites. Previous investigations of cyst sensitivity to disinfectants have been carried out at different times after inducing encystment. However, the results of this study suggest that cyst maturity should be considered more carefully when carrying out studies of disinfectant efficacy. In the past, cyst maturity was thought to be affected by the method used to induce encystment. However, subsequent studies have shown that cysts respond similarly to PHMB irrespective of whether encystment is induced by culture in Neff's constant-pH encystment medium or non-nutrient agar [36, 37]. On the other hand, when Kilvington and Anger [37] examined the sensitivity to MPS of comparatively immature cysts (0.5–7 days from the onset of encystment), they discovered that cysts become progressively less sensitive to the solutions as they mature. Our results and Kilvington and Anger's findings both suggest that cysts remain sensitive to PHMB even after incubation in an encystment medium for 1 week, and only gain resistance to the

disinfectant after undergoing further maturation. Because the maturity of the cysts seems to have a strong effect on their sensitivity to disinfectants, it is necessary to adopt standardized methods of inducing encystment and evaluating cyst maturity when conducting comparative investigations of disinfectant solutions.

The results of this study confirm that many SCL disinfectant solutions commercially marketed in Japan are not adequately effective against *Acanthamoeba*, and cannot be expected to completely disinfect SCL. These findings highlight the importance of warning SCL users about the risks of AK, and thorough education about correct lens care practices. Previous studies indicate that topping up old disinfectant solution, inadequate lens case hygiene, failure to wash the hands before handling SCL, and elimination of rubbing and rinsing steps are risk factors for AK. In particular, rubbing and rinsing should be emphasized to SCL wearers because it can prevent *Acanthamoeba* from adhering to SCL [38]. In addition, further investigation of the inactive ingredients in disinfectant solutions is warranted in order to maximize the efficacy of disinfectants in future products. Objective standards for evaluating disinfectant efficacy against *Acanthamoeba* have not yet been established. However, the log reduction method used in this study yields objective and quantitative results, and should be regarded as a simple and reliable way of evaluating the biocidal activity of disinfectant solutions against *Acanthamoeba.*

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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 immunecompromised 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 Light-Cycler (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

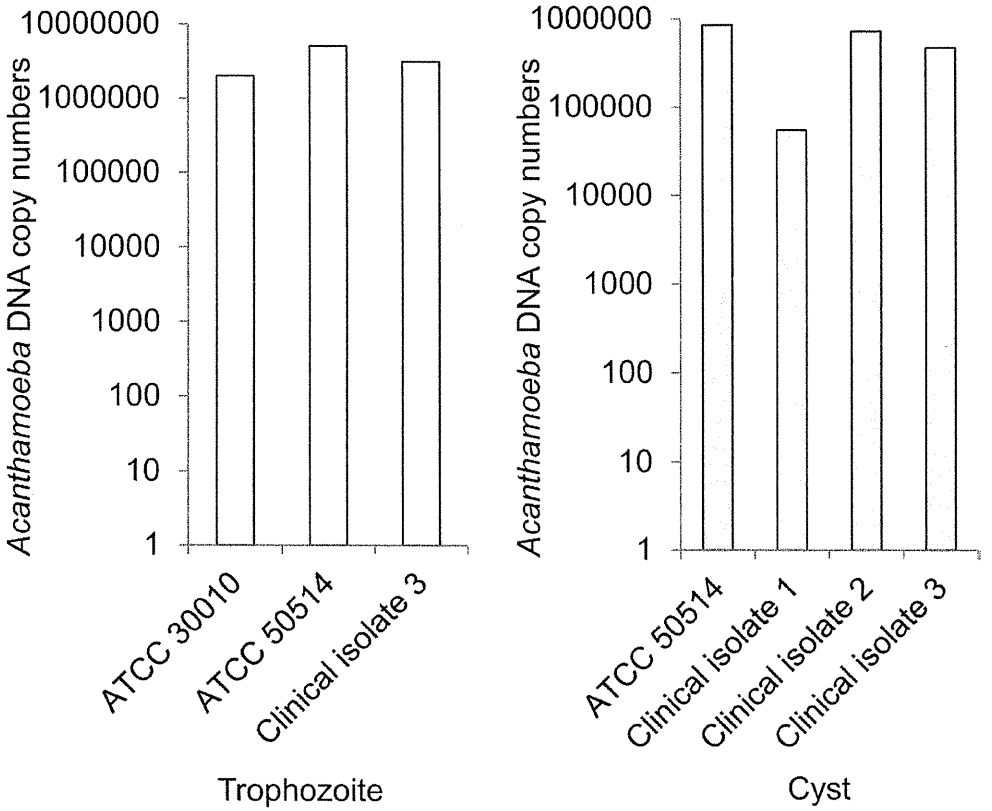

Figure 1. Evaluation of *Acanthamoeba* DNA detection in cultured *Acanthamoeba* cysts and trophozoites. Different strains of *Acanthamoebae* were cultured, and their cysts or trophozoites were diluted to 10 000 counts/ml. The extracted DNA was assessed for the *Acanthamoeba* DNA copy numbers. *Acanthamoeba* 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

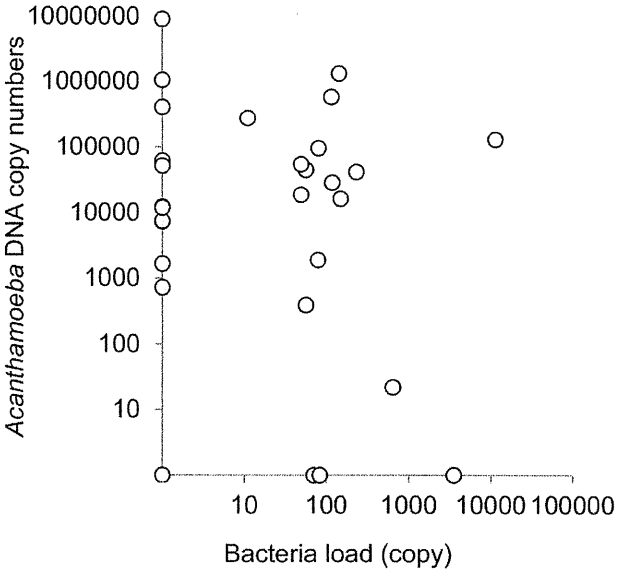

Figure 2. Evaluation of *Acanthamoeba* DNA copy number in *Acanthamoeba* keratitis determined by real-time polymerase chain reaction in relation to bacterial load. *Acanthamoeba* DNA copy number at first visit is not significantly correlated with the bacterial load in the lesion.

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–

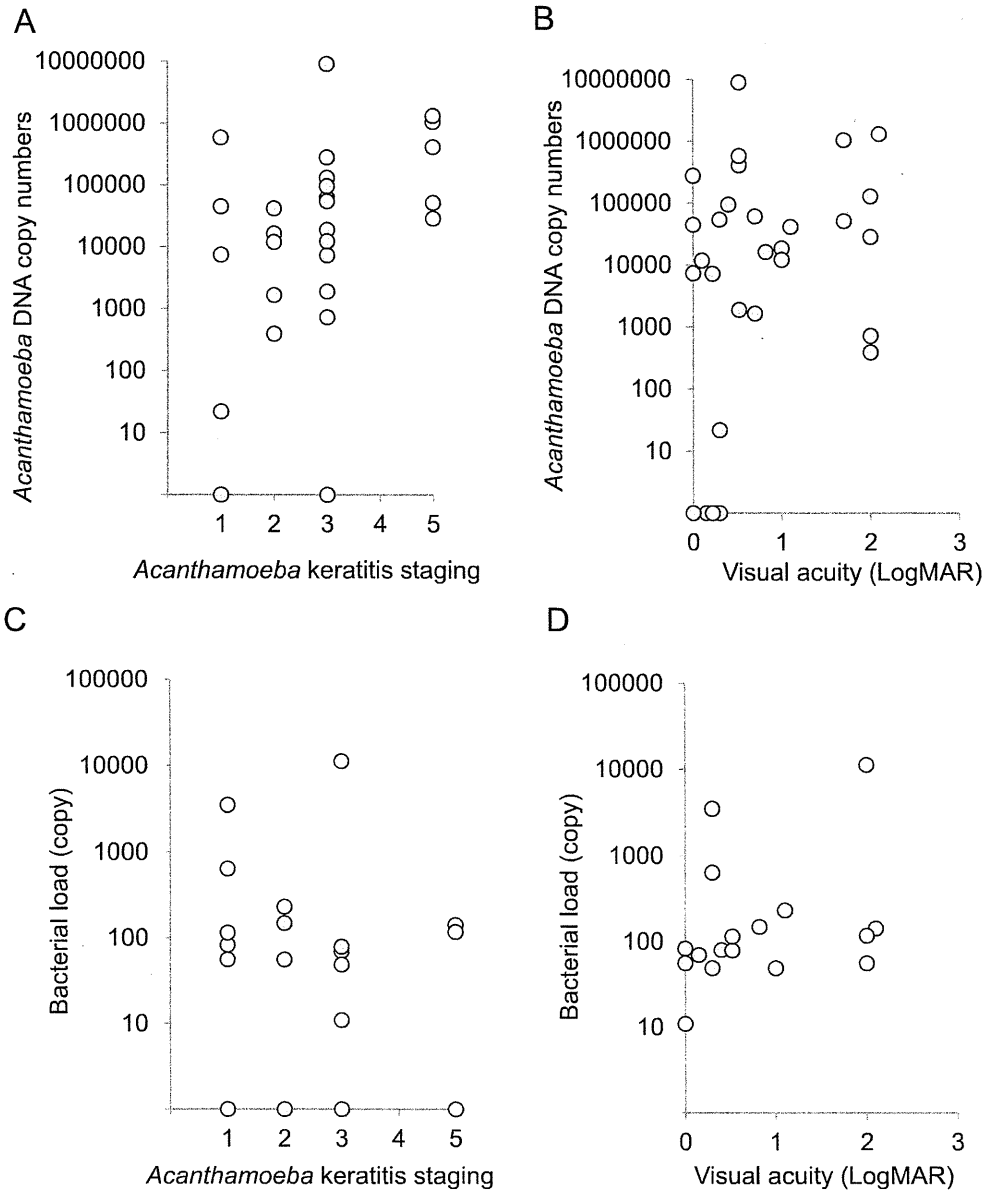

Figure 3. Significant correlation of *Acanthamoeba* DNA copy number with stage of *Acanthamoeba* keratitis (AK) and visual acuity. *Acanthamoeba* DNA copy number at first visit was significantly correlated with AK stage (ρ=0.53) **(A)** and logarithm of the minimum angle of resolution visual acuity (ρI=0.37) **(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-

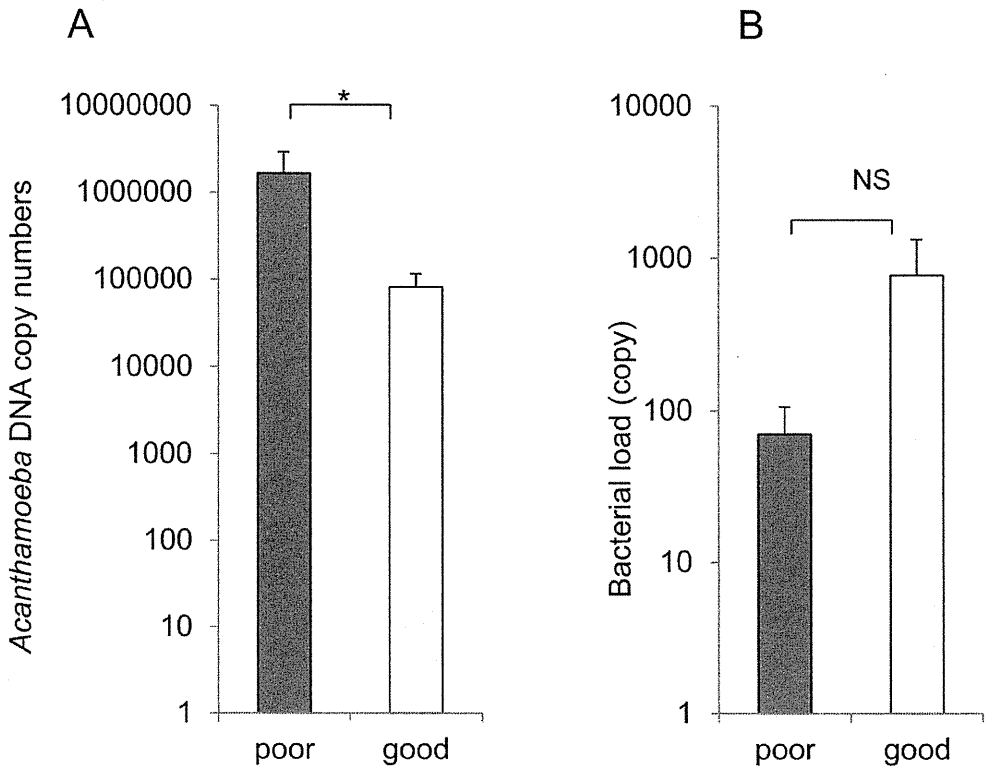

Figure 4. Elevation of *Acanthamoeba* DNA copy number in *Acanthamoeba* keratitis (AK) cases with poor outcomes. The AK cases with poor outcome, defined as visual acuity <20/50 at last visit or requiring keratoplasty, have a significantly higher number of *Acanthamoeba* DNA copies at the first visit 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.

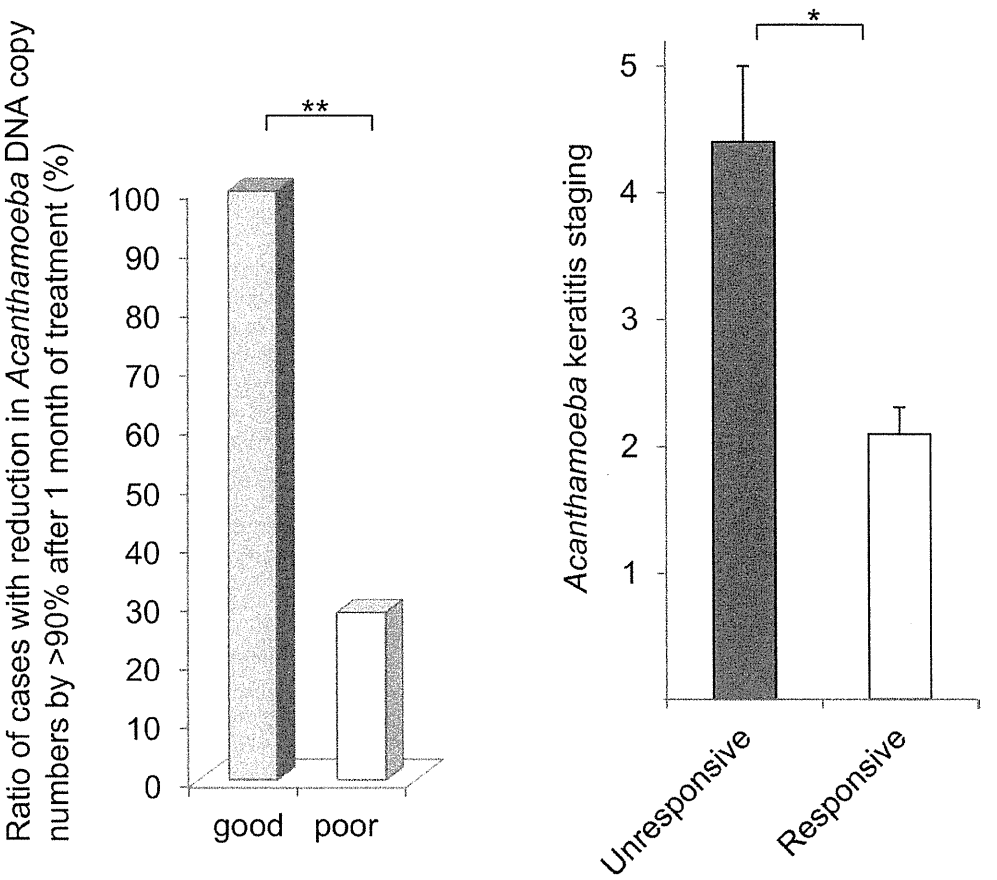

Figure 5. Association of poor outcomes with unresponsive *Acanthamoeba* copy reduction after treatment. Ratio of *Acanthamoeba* keratitis (AK) cases with unresponsive *Acanthamoeba* copy reduction after 1 month of treatment was significantly higher in AK with adverse prognosis **(A)**. In the unresponsive AK cases, AK stage was significantly advanced compared with the responsive cases for amoebic copy reduction **(B)**. $*P<0.01$. $**P=0.0005$.

Acanthamoeba because it does not rely on the functional integrity of the amoeba as do the conventional methods. The high sensitivity of the real-time PCR is also derived from the specificity of the TaqMan probe method[3] and the precise regression to the predetermined amount of amoebic DNA standards.

Even though *Acanthamoeba* is environmentally ubiquitous, our real-time PCR did not detect amoebic DNA in conjunctival smears from normal subjects. Although the number of subjects tested was limited, our findings indicate that amoebic trace is most likely absent in healthy eyes.

Acanthamoeba preys mainly on bacteria but also on fungi and other protozoans. Thus, *Acanthamoeba* might be observed as coinfectants in infectious keratitis cases. When we determined the specificity of *Acanthamoeba* PCR in BK cases, *Acanthamoeba* DNA was not detected in any of the BK cases, but 53.6% of the AK cases had low levels of bacterial DNA. This supports the concept of a bacterial involvement in the cause of AK, although the stage of the AK was not significantly correlated with the bacterial load (Fig 3). Thus, once AK is established, the bacterial load probably plays a limited role in its progression.

Table 2. Parameters Associated with Unresponsive *Acanthamoeba* DNA Reduction after 1 Month by Logistic Regression Analysis

	Odds Ratio					
	Lowest Category	*Second Category*	*95% CI*	*Highest Category*	*95% CI*	*P Value*
AK stage	1.0	Stage 2:8.00	1.06–58.82	Stage 5:4096	1.28–11 973 037	0.04*
Acanthamoeba DNA copy number at the first visit	1.0	≤1000:2.79	0.98–8	>100 000:60.88	0.92–4096	0.055
Bacterial load at the first visit	1.0	≤10:1.30	0.60–2.85	>10 000:6.52	2.99–14.25	0.51

AK = *Acanthamoeba* keratitis.
*$P\leq0.05$.

In the course of lengthy treatments for AK, clinicians are often frustrated when a chosen treatment regimen is ineffective. In refractory cases, *Acanthamoeba* is sometimes resistant to antifungal drugs or antiseptic drugs. Indeed, in our case series, multidrug-resistant *Acanthamoeba* were detected especially in the refractory cases. Moreover, drug-sensitivity testing of *Acanthamoeba* in vitro takes weeks for completion and does not necessarily mirror the sensitivity to the drugs in vivo, especially in refractory cases.[12] This suggests that the proliferation of *Acanthamoeba* seems to depend on both an impaired immune response of the host and the virulence of the *Acanthamoeba.* Consistent with this, the AK outcome was significantly correlated with an unresponsive reduction of amoeba copy numbers after anti-amoeba treatment.

Previous multivariate analysis of AK showed that the duration of the symptoms before diagnosis was a risk factor for a more advanced stage of the disease, and the more advanced stage at presentation was a risk factor for worse outcome.[4] Consistent with these findings, advanced disease stage was one of the significant risk factors for poor outcomes. Furthermore, we found that the detected *Acanthamoeba* copy numbers at the first visit were another risk factor. Advanced AK stage was also a risk factor for unresponsive reduction of amoebic DNA.

Our findings should help clinicians make earlier decisions on when to switch to surgical intervention after treatment. Of note, risk assessments for poor outcomes do not necessarily require real-time amoebic PCR. We suggest that conventional PCR or even smear staining would be sufficient for this purpose. For example, careful sampling of AK lesions during the course of treatment and evaluations by conventional Calcofluor or Fungiflora Y staining will determine whether more than 90% of amoebic bodies have been cleared after 1 month of treatment.

The sensitivity of real-time PCR in patients with AK did not reach the theoretic 100% sensitivity that real-time PCR should have achieved, perhaps because the sampled amount was not sufficient and the sampled location was not correct. The staining of corneal lesions usually requires more tissues, and therefore staining samples were collected before sampling for PCR. When AK is at an early stage and has low amoebic numbers, the sampling may remove even trace amounts of *Acanthamoeba.* In this case, smear staining would be positive but PCR would be negative. The location or depth of the lesion may also affect its outcome. For example, when samples are obtained from inflammatory-prone lesions at the early stage, but without amoeba, real-time PCR would be negative.

The strong immune responses of the host also affect the amoebic DNA load. Aggressive AK treatment or presumably host factors would exacerbate the *Acanthamoeba* copy numbers. This can present as dense inflammatory opacities that are difficult to differentiate from AK with high levels of *Acanthamoeba.* Indeed, we had a case with low visual acuity due to severe corneal and anterior chamber inflammation, in which the small amount of amoebic DNA was readily eradicated after a few weeks of treatment, and treatment was successfully switched to topical steroid therapy to reduce the inflammatory responses.

Refractory AK cases sometimes require therapeutic keratoplasty. The management of post-keratoplasty cases requires intensive use of steroids because they are susceptible to rejection because of the larger graft size and strong inflammatory environment provoked by the AK.

Real-time PCR for *Acanthamoeba* is also useful for confirmation of the complete removal of *Acanthamoeba.* It is a great relief for surgeons to know that the amoebic DNA becomes negative after surgical intervention in cases with advanced-stage AK with a million copies. *Acanthamoeba* real-time PCR requires only a minute amount of sample and is useful for confirming the absence of *Acanthamoeba.* Amoebic PCR ensures the validity of aggressive treatment or surgical intervention and would support the proper timing for the use of steroids for better visual outcome.

In conclusion, collectively, *Acanthamoeba* real-time PCR is effective in diagnosing AK. Real-time PCR detection does not provide information on virulence of *Acanthamoeba* or immunologic responses of the host, but it does provide useful information in managing AK.

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Footnotes and Financial Disclosures

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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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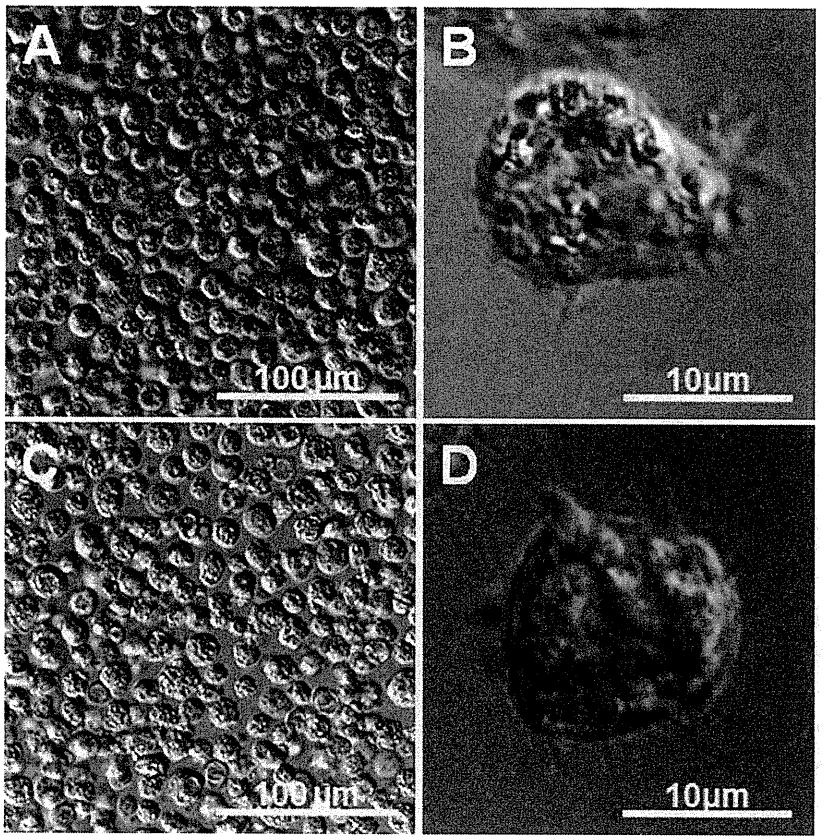

FIG 1 *Acanthamoeba castellanii* trophozoites stained with CTC. Merged fluorescence images of red-fluorescing CTC formazan and differential interference contrast images of trophozoites stained with CTC are shown. (A) CTC formazan accumulates within trophozoites, as shown by red fluorescence. (B) Trophozoite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

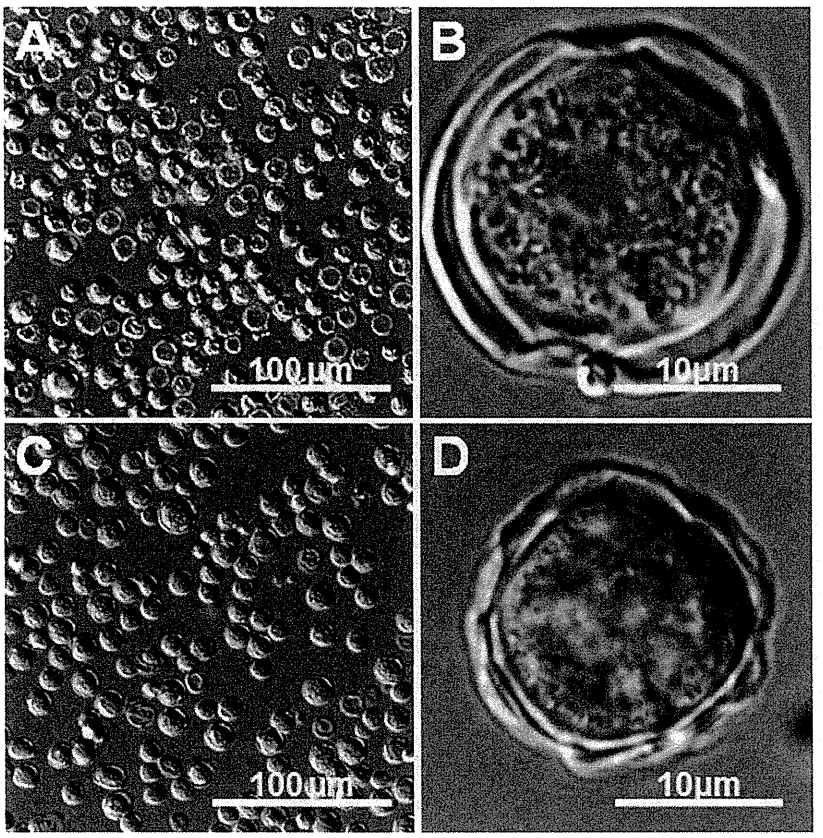

FIG 2 *Acanthamoeba castellanii* cysts stained with CTC. Merged fluorescence images of red-fluorescing CTC formazan and differential interference contrast images of cysts stained with CTC are shown. (A) CTC formazan accumulates within cysts, as shown by red fluorescence. (B) Cyst from panel A at higher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

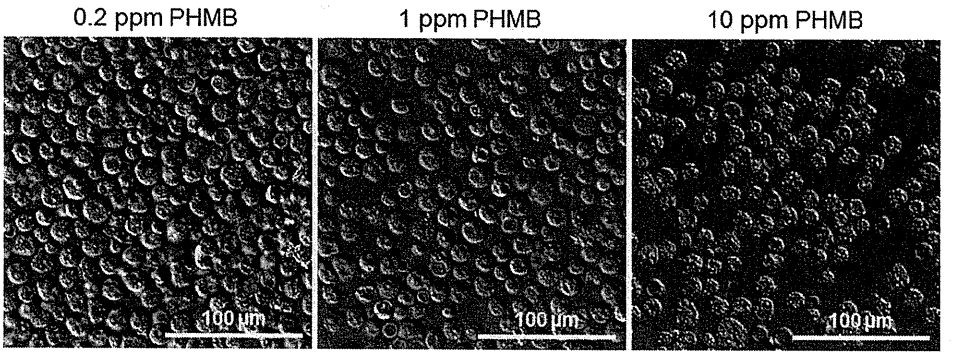

FIG 3 CTC staining of PHMB-treated *Acanthamoeba* trophozoites. Merged fluorescence images of red-fluorescing CTC formazan and differential interference contrast images of trophozoites stained with CTC after PHMB treatments are shown. CTC staining was performed on trophozoites treated with PHMB (0.2,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

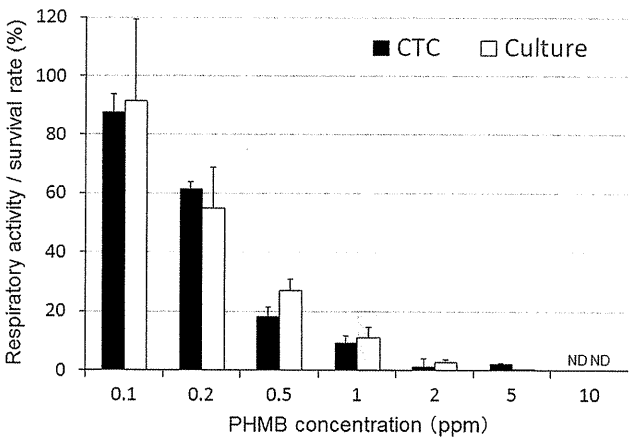

FIG 4 Respiratory activity and survival rate of *Acanthamoeba* trophozoites after PHMB treatment. The respiratory activity (CTC biocidal assay) was compared with the survival rate (culture-dependent biocidal assay) for trophozoites after treatment with PHMB (25°C, 4 h). Error bars represent the standard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

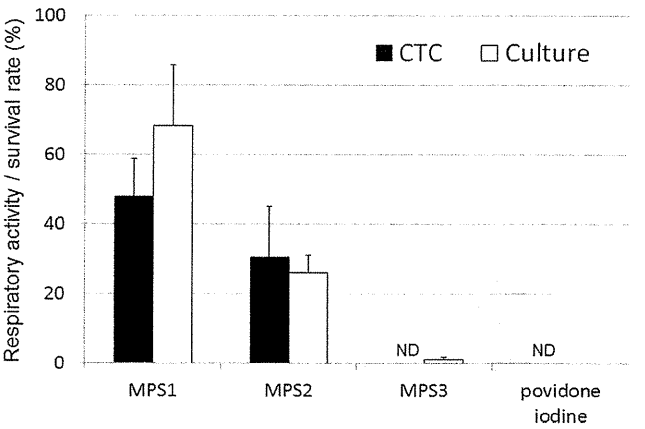

FIG 5 Respiratory activity and survival rate of *Acanthamoeba* trophozoites after treatment with SCL disinfectant solutions. The respiratory activity (CTC biocidal assay) was compared with the survival rate (culture-dependent biocidal assay) for trophozoites after treatment with SCL disinfectant solutions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

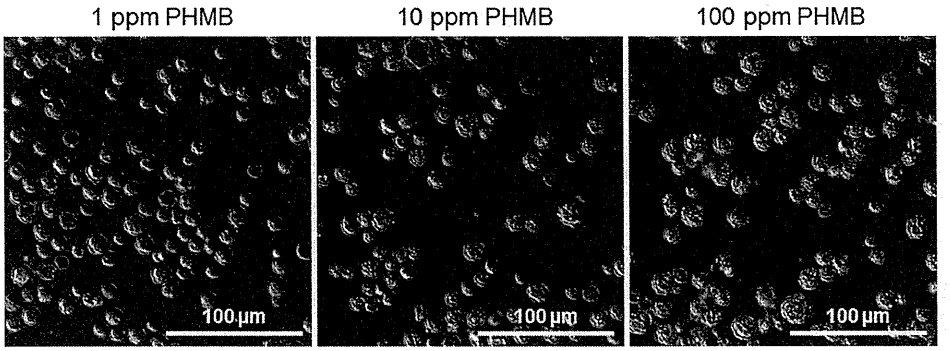

FIG 6 CTC staining of PHMB-treated *Acanthamoeba* cysts. Merged fluorescence images of red-fluorescing CTC formazan and differential interference contrast images of cysts stained with CTC after PHMB treatments are shown. CTC staining was performed on cysts treated with PHMB (1, 10, or 100 ppm) for 4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

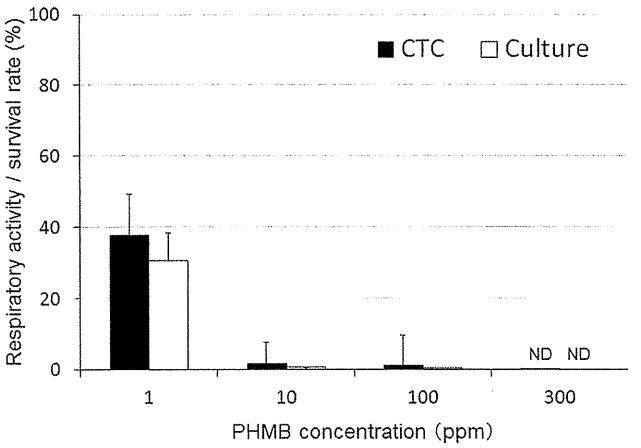

FIG 7 Respiratory activity and survival rate of *Acanthamoeba* cysts after PHMB treatment. The respiratory activity (CTC biocidal assay) was compared with the survival rate (culture-dependent biocidal assay) for cysts after treatment with PHMB (25°C, 4 h). Error bars represent the standard errors of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

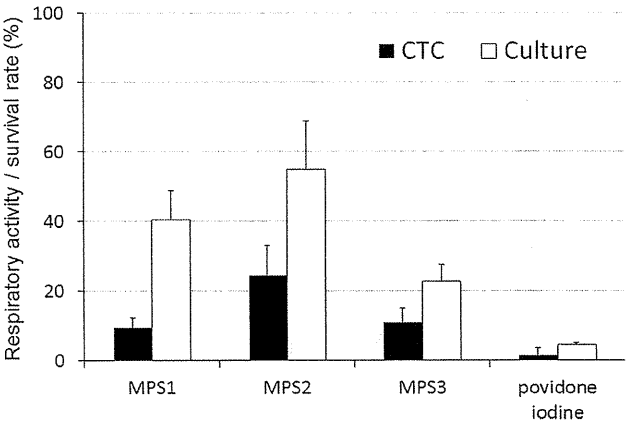

FIG 8 Respiratory activity and survival rate of *Acanthamoeba* cysts after treatment with SCL disinfectant solutions. The respiratory activity (CTC biocidal assay) was compared with the survival rate (culture-dependent biocidal assay) for cysts after treatment with SCL disinfectant solutions (25°C, 4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