Acanthamoeba polyphaga Strain Age and Method of Cyst Production Influence the Observed Efficacy of Therapeutic Agents and Contact Lens Disinfectants

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The effects of age in culture and the type of medium used for induction of *Acanthamoeba polyphaga* (Ros) cysts on susceptibilities to polyhexamethylene biguanide (PHMB; 3 μg/ml), chlorhexidine digluconate (30 μg/ml), myristamidopropyl dimethylamine (20 μg/ml), H_2O_2 (3%), and two multipurpose contact lens solutions (MPS-1 and MPS-2, based on 1 μg of PHMB per ml) were examined. Strain Ros-02 was cryopreserved on isolation in 1991, while strain Ros-91 had been in continuous axenic culture. Significant differences in susceptibilities to the disinfectants were found depending on the medium used for cyst preparation and the age of the test strain, with Ros-02 generally being more resistant. For example, the killing of Ros-91 cysts produced from an axenic culture of trophozoites in the presence of 50 mM $MgCl_2$ by MPS-2 was 4 logs, but the killing of Ros-02 by MPS-2 was only 2 logs ($P < 0.05$) and killing of both strains with cysts obtained from monoxenic cultures with *Escherichia coli* was only 1 log ($P < 0.001$). Assays repeated with different batches of the various cyst types gave consistent results. A batch of Ros-91 cysts stored at 4°C and tested over an 8-week period with MPS-1 showed progressively increasing susceptibility to disinfection, although there was no loss of viability during storage ($P < 0.01$). These observations have important implications for the standardization and interpretation of *Acanthamoeba* disinfectant and therapeutic agent testing.

Acanthamoeba is a genus of small free-living amoeba characterized by a life cycle of feeding and replicating trophozoite and dormant cyst stages (25). The resistance of the cyst stage to extremes of temperature, desiccation, and disinfection accounts for the wide distribution of *Acanthamoeba* in most soil and aquatic habitats (15, 25). *Acanthamoeba* is pathogenic for humans, causing rare but fatal encephalitis in the immunocompromised host and, more frequently, a potentially blinding infection of the cornea in previously healthy persons (19). Contact lens wearers are most at risk from infection and account for some 90% of all reported cases in the Western Hemisphere (26, 27). Failure to comply with recommended lens cleaning and disinfection instructions and the rinsing or storage of lenses in nonsterile saline solutions or tap water are recognized risk factors (28, 33).

The relative ease with which most *Acanthamoeba* species and strains can be adapted and maintained in axenic culture has made the organism an ideal model for cellular and molecular biological studies, notably, the process of differentiation into the cyst stage (7, 18, 22). In the laboratory, encystment can be induced by starvation through depletion of the bacterial food source from prolonged culture of trophozoites on nonnutrient agar (NNA) seeded with *Escherichia coli* or in axenic medium, often for up to 6 weeks or more (4, 5, 25). In an attempt to control the process under defined conditions, media that promote rapid and synchronous encystment have been

developed. These include Neff's constant-pH encystment medium containing the divalent cations Mg^{2+} and Ca^{2+} and amine buffer at a pH of 8.9 to 9.0 or medium with 50 mM $MgCl_2$, which is added to axenically growing trophozoite cultures (12, 22). Incubation with the amino acid taurine and Mg^{2+} has also been shown to induce encystment (32).

The increasing incidence of *Acanthamoeba* keratitis among contact lens wearers has necessitated the evaluation and development of potential therapeutic agents and contact lens disinfectants active against the resistant cyst stage of the organism (3, 5, 13, 26, 33). Disinfection is fundamental to safe contact lens use and, hence, the prevention of infection. This is commonly achieved through the use of multipurpose solutions, in which a single solution is used to disinfect, clean, and store lenses, or with hydrogen peroxide-based systems (5, 9, 30). Unlike the requirements for bacteria and fungi, no standard protocol exists for testing of the efficacies of disinfectants against *Acanthamoeba* for use with contact lenses (11). As a consequence, a variety of strains, methods for cyst production, and experimental protocols have been used (1, 5, 9, 10, 13, 24, 31), often with contradictory findings (2, 6, 8, 12, 23).

In an attempt to develop standardized and reproducible methods for the evaluation of contact lens disinfectants and therapeutic agents for their activities against *Acanthamoeba*, we have compared the effects of age in culture and the method of cyst production on the efficacies of contact lens disinfectants and experimental agents against a strain of *Acanthamoeba polyphaga* originally isolated from a case of keratitis in 1991. The finding that these experimental variables can significantly affect the observed efficacies of disinfectant and therapeutic solutions against *Acanthamoeba* has prompted this report.

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TABLE 1. Effects of strain age and method of cyst production on *A. polyphaga* disinfection

Drug	Strain[a]	Log killing (mean ± SEM)[b]							
		6-Mg		Neff		Taurine		NNA	
		4h	6h	4h	6h	4h	6h	4h	6h
PHMB (3 μg/ml)	Ros-91	4.0 ± 0.2	4.0 ± 0.2	1.7 ± 0.7	3.1 ± 0.2	0.4 ± 0.3	0.4 ± 0.3	2.6 ± 0.4	3.3 ± 0.2
	Ros-02	0.9 ± 0.4	3.4 ± 0.6	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.8 ± 0.1	1.2 ± 0.3
MAPD (20 μg /ml)	Ros-91	3.9 ± 0.1	3.9 ± 0.1	3.7 ± 0.1	3.7 ± 0.1	2.9 ± 0.4	3.8 ± 0.1	1.7 ± 0.2	2.8 ± 0.3
	Ros-02	3.9 ± 0.1	3.9 ± 0.1	2.1 ± 0.2	4.5 ± 0.1	1.8 ± 0.0	2.9 ± 0.3	1.7 ± 0.2	2.7 ± 0.2
MPS-1 (1 μg /ml)	Ros-91	3.6 ± 0.4	4.3 ± 0.1	2.8 ± 0.2	3.9 ± 0.6	2.8 ± 0.1	3.7 ± 0.4	1.6 ± 0.3	1.7 ± 0.3
	Ros-02	3.0 ± 0.4	3.8 ± 0.1	1.9 ± 0.5	3.4 ± 0.2	1.2 ± 0.3	1.5 ± 0.3	0.9 ± 0.2	1.7 ± 0.4
MPS-2 (1 μg /ml)	Ros-91	1.8 ± 0.4	3.7 ± 0.4 (2.9 ± 0.5)[c]	0.8 ± 0.1	1.0 ± 0.0 (1.4 ± 0.2)	2.4 ± 0.3	3.3 ± 0.4	0.4 ± 0.2	1.0 ± 0.2 (1.1 ± 0.3)
	Ros-02	1.7 ± 0.2	2.4 ± 0.1	1.2 ± 0.2	1.8 ± 0.3	1.1 ± 0.1	1.3 ± 0.0	0.2 ± 0.1	1.0 ± 0.1
CHLX (30 μg /ml)	Ros-91	0.8 ± 0.1	1.0 ± 0.3	1.7 ± 0.3	1.8 ± 0.3	0.5 ± 0.1	0.8 ± 0.2	0.8 ± 0.1	1.5 ± 0.5
	Ros-02	0.7 ± 0.2	1.2 ± 0.5	0.6 ± 0.4	1.1 ± 0.2	0.1 ± 0.1	0.4 ± 0.1	0.2 ± 0.3	0.2 ± 0.3
Peroxide (3%)	Ros-91	2.9 ± 0.3	3.8 ± 0.1	2.4 ± 0.1	2.7 ± 0.1	2.1 ± 0.2	3.5 ± 0.5	2.5 ± 0.2	3.9 ± 0.5
	Ros-02	3.3 ± 0.6	4.2 ± 0.2	1.7 ± 0.3	4.3 ± 0.1	2.5 ± 0.2	3.4 ± 0.6	0.9 ± 0.3	2.8 ± 0.5
One-quarter-strength Ringer's (control)	Ros-91	0.1 ± 0.0	0.0 ± 0.0	0.2 ± 0.3	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.2	0.2 ± 0.2	0.0 ± 0.1
	Ros-02	0.2 ± 0.1	0.0 ± 0.2	0.3 ± 0.2	0.2 ± 0.4	0.0 ± 0.0	0.0 ± 0.1	0.1 ± 0.1	0.1 ± 0.2

[a] *A. polyphaga* strain Ros-02 was cryopreserved shortly after isolation in 1991 and was recovered for this study; strain Ros-91 has been in continuous axenic culture since that time.
[b] The experiments were conducted in triplicate.
[c] The values in parentheses are the log killings obtained with additional batches of Ros-91 cysts tested on a separate occasion.

MATERIALS AND METHODS

***A. polyphaga* strains, culture, and cyst preparation.** *A. polyphaga* strain Ros-02 was cryopreserved at the time of isolation in 1991 from a patient with *Acanthamoeba* keratitis (16). Strain Ros-91 was the same isolate but had been maintained in continuous axenic broth culture since 1991. Trophozoites were adapted and maintained in a semidefined axenic broth (medium 6), as described previously (9). Ros-02 was passaged no more than five times before being used for cyst production.

Cysts of both strains were prepared from the trophozoites in the following manner: (i) by culture on 2% NNA in one-quarter-strength Ringer's solution (Oxoid Ltd., Basingstoke, England) seeded with live *E. coli* (NNA cysts) in air at 32°C for 7 days; (ii) by culture with Neff's constant-pH encystment medium (Neff cysts), as described previously (9, 22); (iii) by culture in one-quarter-strength Ringer's solution containing 20 mM taurine and 15 mM MgCl$_2$ (Sigma Chemical Company, Dorset, England) under the same conditions described for the preparation of the Neff cysts (taurine cysts); and (iv) by growing trophozoites in semidefined axenic medium (medium 6) supplemented after autoclaving with filter-sterilized 50 mM MgCl$_2$ (6-Mg), again, with the same incubation conditions described previously (9, 12). The cysts were harvested and washed three times with one-quarter-strength Ringer's solution by centrifugation at $1,000 \times g$ for 5 min and stored at 4°C for testing within 7 days.

A single batch of each cyst preparation was used in all experimental studies. To determine the reproducibilities of the cyst preparations, four different batches of Ros-91 6-Mg cysts were prepared and tested with multipurpose contact lens disinfection solution no. 1 (MPS-1) on separate occasions. In addition, different batches of Ros-91 6-Mg, Neff, and NNA cysts were also made and retested with MPS-2. To assess the effect of storage on cyst susceptibility, a batch of Ros-91 6-Mg cysts was prepared and stored at 4°C for testing with MPS-1 over an 8-week period.

Flow cytometry and microscopy. Unstained cyst preparations were compared with a FACSCalibur flow cytometer (Becton Dickinson Bioscience, San Jose, Calif.). Data for at least 2,000 events were collected, and the forward scatters were plotted to compare the cell sizes of the preparations. The findings were analyzed with CellQuest Pro (version 4.0.1) software. The unstained cyst preparations were observed by phase-contrast microscopy. Batches of Ros-91 6-Mg and Neff cysts were also examined by transmission electron microscopy.

Test solutions. The following contact lens disinfectants and experimental agents were studied: 3% hydrogen peroxide (contact lens disinfection solution), polyhexamethylene biguanide (PHMB) at 3 μg/ml in H$_2$O (Bausch & Lomb, Rochester, N.Y.), chlorhexidine digluconate (CHLX) at 30 μg/ml in H$_2$O (Sigma Chemical Company), the amidoamine myristamidopropyl dimethylamine (MAPD) at 20 μg/ml in 2 mM Tris·HCl (pH 7.2; Alcon Laboratories Inc., Fort Worth, Tex.), and MPS-1 and MPS-2, both of which contained 1 μg of PHMB per ml. In control experiments, one-quarter-strength Ringer's solution was used in place of the test solutions.

Cysticidal assay. The method used to determine the kinetics of cyst killing was described previously (9, 13). Briefly, 100 μl of 2×10^6 *A. polyphaga* (Ros) cysts per ml were inoculated into 10 ml of test or control solution in 50-ml polypropylene centrifuge tubes (Becton Dickinson, Oxford, England). For all test solutions except hydrogen peroxide, the tubes were aged overnight by filling them with 10 ml of the test solution, which was then discarded and replaced with fresh solution for testing. At time intervals of 0, 1, 2, 4, and 6 h, the solution was vortexed and 20 μl was removed in quadruplet and added to 200 μl of 4 mg of bovine liver catalase (Sigma Chemical Company) per ml for neutralization of the hydrogen peroxide or 0.1% Tween 80 (Sigma Chemical Company) for neutralization of the other disinfectants in the wells of a 96-well flat-bottom microtiter plate (Triple Red Laboratory Technology, Oxford, United Kingdom). After 5 min, serial dilutions of 20 μl in 200 μl of one-quarter-strength Ringer's solution (Oxoid) were then made across the rows of the microtiter plate in quadruplet. Twenty-five microliters of *E. coli* JM101 (optical density at 600 nm, 0.4) was then added to each well containing the cyst dilutions, and the plate was sealed and incubated at 32°C for up to 7 days. Viable cysts hatch (excyst) in the presence of the live *E. coli* isolates, and the subsequent growth and replication of the trophozoite can be observed microscopically. The plates were inspected daily for 7 days for the presence or absence of excystment and trophozoite replication in the wells. This enables the number of cysts killed to be assessed by a most probable number approach (4, 29).

Data analysis. The number of surviving cysts in the time-kill studies was determined by using the computations of Reed and Muench (29), as described previously for *Acanthamoeba* (4). The reduction in organism viability was plotted as the change in log viability at each time point compared to the viability at zero time. Statistical analysis was performed by one-way analysis of variance (ANOVA) from mean ± standard error of the mean (SEM) values from triplicate experiments.

RESULTS

The efficacies of the disinfectants against the various preparations of *A. polyphaga* Ros-91 and Ros-02 cysts are shown in Table 1, which gives the mean ± SEM log cyst killings from triplicate experiments. Only the findings obtained after 4 and

TABLE 2. Statistical comparison (ANOVA) of *A. polyphaga* strains (Ros-91 and Ros-02) and different cyst preparations against disinfectant agents

| Strain | Statistical significance[a] | | | | | | | | | | | | | | | | | | | | |
| | PHMB (3 μg/ml) | | | | | | | MAPD (20 μg/ml) | | | | | | | MPS-1 | | | | | | |
	Mg-91	Mg-02	Neff-91	Neff-02	Taur-91	Taur-02	NNA-91	Mg-91	Mg-02	Neff-91	Neff-02	Taur-91	Taur-02	NNA-91	Mg-91	Mg-02	Neff-91	Neff-02	Taur-91	Taur-02	NNA-91
Mg-02	***/NS							NS/NS							NS/NS						
Neff-91	**/NS	NS/NS						NS/NS	NS/NS						NS/NS	NS/NS					
Neff-02	***/***	NS/***	NS/***					***/NS	***/NS	***/*					*/NS	NS/NS	NS/NS				
Taur-91	***/***	NS/***	NS/***	NS/NS				*/NS	*/NS	NS/NS	NS/NS				NS/NS	NS/NS	NS/NS	NS/NS			
Taur-02	***/***	NS/***	NS/***	NS/NS	NS/NS			***/*	***/*	***/NS	NS/***	*/*			**/***	*/**	*/**	NS/*	*/**		
NNA-91	NS/NS	NS/NS	NS/NS	**/***	**/***	**/***		***/**	***/*	***/*	NS/***	**/*	NS/NS		**/**	NS/**	NS/**	NS/*	NS/*	NS/NS	
NNA-02	***/***	NS/*	NS/*	NS/NS	NS/NS	NS/NS	*/**	***/**	***/*	***/*	NS/***	*/**	NS/NS	NS/NS	***/**	***/**	*/**	NS/*	*/*	NS/NS	NS/NS

[a] Statistical significance at 4 and 6 h: NS, not significant ($P > 0.05$); *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. 91, Ros-91; 02, Ros-02; Mg, 6 Mg medium; Neff, Neff's medium; Taur, taurine Mg medium; NNA, nonnutrient agar *E. coli* medium.

6 h of exposure are shown, as little significant killing occurred before these time points. All control experiments with one-quarter-strength Ringer's solution gave ≤0.3-log killing (Table 1). Comparative statistical analysis (ANOVA) of the experimental findings are summarized in Table 2. Although the patterns of resistance varied between the test solutions, significant differences in susceptibility occurred depending on the cyst preparation and the age of the test strain (Tables 1 and 2).

For example, with PHMB (3 μg/ml), 3- to 4-log killing of 6-Mg, Neff, and NNA cysts of Ros-91 was shown at 6 h, yet only 0.4-log killing of taurine cysts was detected ($P < 0.001$). Three-log killing was also obtained for the Ros-02 6-Mg cysts, yet the log killing values for the Neff, taurine, and NNA cysts were 0.3, 0.2, and 1.2, respectively ($P < 0.01$).

MAPD (20 μg/ml) was active against all Ros-91 cyst types, giving 2.8- to 4-log killing at 6 h, although the value of 2.8-log killing for the NNA cyst was significantly lower than those for the other cyst types ($P < 0.01$). With Ros-02, the values ranged from 2.7- to 4.5-log killing at 6 h, with the decreased values of 2.9 and 2.7 logs for the taurine and NNA cysts being significant with respect to the values for the 6-Mg and Neff cysts ($P < 0.05$ and $P < 0.01$, respectively).

MPS-1 and MPS-2, both based on 1 μg of PHMB per ml, varied in their cysticidal efficacies. Treatment with MPS-1 resulted in only 1.7-log killing at 6 h for Ros-91 NNA cysts, whereas 4-log killing was found for the rest of the Ros-91 cyst types ($P < 0.01$). For Ros-02, 3.8- and 3.4-log killings were observed for the 6-Mg and Neff cysts, respectively, whereas 1.5- and 1.7-log killings were observed for the taurine and NNA cysts, respectively ($P < 0.01$).

Treatment with MPS-2 resulted in 3.3- to 3.7-log killing of Ros-91 taurine and 6-Mg cysts at 6 h but only 1-log killing of Ros-91 Neff and NNA cysts ($P < 0.001$). In contrast, 2.4-log killing was obtained for the Ros-02 6-Mg cysts at 6 h, whereas 1.0- to 1.8-log killings were obtained for the other Ros-02 cyst preparations ($P < 0.05$). The greater sensitivity of the Ros-91 6-Mg cysts than the Ros-02 6-Mg cysts was also significant ($P < 0.05$).

CHLX (30 μg/ml) had only low cysticidal activity, giving 0.8- to 1.8-log killing of the Ros-91 cyst preparations and 0.1- to 1.1-log killing of the Ros-02 cyst preparations after 6 h; none of the findings at 6 h showed significant differences ($P > 0.05$). H_2O_2 (3%) showed a greater consistency of activity against all cyst types and strains of both ages, giving at least a 2.7- to 4.3-log killing after 6 h of exposure, with no significant differences observed between cyst types or strains at 6 h.

The four separate batches of Ros-91 6-Mg cysts tested with MPS-1 had log cyst killings after 4 h of exposure of 2.6 ± 0.7, 3.1 ± 0.4, 3.0 ± 0.6, and 1.8 ± 0.5, respectively. No significant difference in susceptibility was found between the cyst batches ($P > 0.05$). Different batches of Ros-91 6-Mg, Neff, and NNA cysts tested with MPS-2 had log killings at 6 h that were consistent with the results obtained with the other batches of these cyst preparations tested with MPS-2 (Table 1).

The batch of Ros-91 6-Mg cysts stored at 4°C for testing over an 8-week period with MPS-1 showed progressively increasing susceptibility to disinfection, although there was no loss of viability during storage ($P > 0.05$). With the freshly prepared cysts, a log killing value of 1.4 ± 0.3 was obtained after 4 h of exposure. However, after 1, 2, 4, and 8 weeks storage, the log killing values were 2.0 ± 0.0, 3.6 ± 0.1, 3.9 ± 0.2, and 3.2 ± 0.3, respectively. This increasing susceptibility to disinfection of the cysts on storage was significant for weeks 2 to 8 ($P < 0.01$).

Fluorescence-activated cell sorter analysis of the preparations showed differences in relative cyst size ($P < 0.001$), with the exception of the sizes of the Ros-91 NNA and Ros-91 Neff cysts. Overall, the differences in the sizes between the Ros-02 and Ros-91 cysts were significantly different ($P < 0.001$), although these differences did not correlate with susceptibility or resistance to disinfection (Tables 1 and 2). Phase-contrast microscopy of the various cyst preparations showed that they had similar morphologies, with >90% being in the mature form (results not shown). Transmission electron microscopy of the Ros-91 6-Mg and Neff cysts showed no difference in the morphologies or thicknesses of the cyst walls (results not shown).

DISCUSSION

Unlike testing of the efficacies of contact lens disinfectants against bacteria and fungi, no such requirement or standard protocol exists for testing of the efficacies against *Acanthamoeba* (11, 17). As a consequence, a variety of species, strains, methods of cyst preparation, and experimental protocols have been used, frequently with contradictory findings, as reviewed by Buck et al. (6). In an attempt to develop a standardized, reproducible method for assessment of the cysticidal efficacies of therapeutic agents and contact lens solutions, we compared the effects of age in culture and method of cyst production with *A. polyphaga*. The demonstration that these variables can significantly affect the susceptibilities of *Acanthamoeba* cysts to disinfection has important implications for the testing and in-

TABLE 2—*Continued*

MPS-2							CHLX (30 µg/ml)							Hydrogen peroxide (3%)						
Mg-91	Mg-02	Neff-91	Neff-02	Taur-91	Taur-02	NNA-91	Mg-91	Mg-02	Neff-91	Neff-02	Taur-91	Taur-02	NNA-91	Mg-91	Mg-02	Neff-91	Neff-02	Taur-91	Taur-02	NNA-91
NS/*							NS/NS							NS/NS						
NS/***	NS/*						NS/NS	NS/NS						NS/NS	NS/NS					
NS/**	NS/NS	NS/NS					NS/NS	NS/NS	*/NS					NS/NS	*/NS	NS/NS				
NS/NS	NS/NS	**/***	NS/*				NS/NS	NS/NS	*/NS	NS/NS				NS/NS	NS/NS	NS/NS	NS/NS			
NS/***	NS/*	NS/NS	NS/NS	*/***			NS/NS	NS/NS	**/NS	NS/NS	NS/NS			NS/NS	NS/NS	NS/NS	NS/NS	NS/NS		
*/***	*/NS	NS/NS	NS/NS	***/***	NS/NS		NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS		NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	NS/NS	
/*	**/*	NS/NS	NS/NS	***/***	NS/NS	NS/NS	NS/NS	NS/NS	**/NS	NS/NS	NS/NS	NS/NS	NS/NS	**/NS	**/NS	NS/NS	NS/NS	NS/NS	*/NS	*/NS

terpretation of the cysticidal efficacies of disinfectant and therapeutic agents.

A variety of methods for the production of *Acanthamoeba* cysts have been described (6), and these methods were compared in this study. Axenic culture of trophozoites in semidefined medium supplemented with up to 50 mM Mg^{2+} is commonly used for this purpose. However, the findings of this study indicate that cysts derived by this approach are more susceptible to killing by PHMB and, to a lesser degree, MAPD. Both of these agents are used in multipurpose contact lens solutions, and this may explain in part the discrepancies in the reported cysticidal efficacies of such disinfectants when different methods of cyst production are used (5, 6, 14, 35). The reasons for the increased susceptibility of the Mg^{2+}-derived cyst preparation are unclear. All cysts appeared to have a similar, mature morphology by light and electron microscopy, although flow cytometry analysis suggested differences in the relative sizes of the cysts from the different cyst preparations. However, this did not correlate with susceptibility or resistance to disinfection. Whether encystment induced from the presence of Mg^{2+} ions results in a different chemical composition of the cyst wall that allows greater penetration of these agents warrants further investigation.

It is also apparent that cysts derived from a laboratory strain that has been in prolonged axenic culture are more susceptible to disinfection than those derived from the original isolate. This variable is addressed in the standard protocol for assessing the efficacies of contact lens disinfectant solutions against bacteria and fungi. That protocol requires that the test strains be passaged from the original culture no more that five times prior to testing (11). Laboratory culture is known to induce biological changes in *Acanthamoeba*, such as a loss of virulence and decreases in cellular enzyme activity (20, 21). Such changes may also be reflected in the biochemical composition of the cyst wall, resulting in a greater susceptibility to disinfection.

Other factors also influence the susceptibilities of *Acanthamoeba* cysts to disinfection. Here it was found that Ros-91 6-Mg cysts displayed increasing susceptibility to disinfection by MPS-1 during storage at 4°C, although the culture viability of the cysts remained unchanged during this period. *Acanthamoeba* cysts have been shown to remain viable at 4°C for at least 24 years (20). The storage times for *Acanthamoeba* cysts prior to use in disinfection assays is not always stated but have ranged from 1 to 6 weeks and highlight another experimental variable that may significantly affect disinfectant efficacy findings (9, 10). Although the cyst preparations used here were of

the mature form, it has also been demonstrated that immature cysts are more susceptible to disinfection, which also stresses the importance of using homogeneous cyst populations of known maturity when conducting such assays (14, 34).

Acanthamoeba encystment is a physiological response to adverse environmental changes, such as depletion of the bacterial food source, increasing anaerobiasis, osmolarity, and the presence of sublethal concentrations of certain therapeutic and biocidal agents (7, 15, 18, 25). In vitro, the process is characterized by discrete stages of trophozoite rounding, early cyst wall synthesis of the immature cyst, and mature cyst formation (7, 22, 36). The composition of the *Acanthamoeba* cyst wall has not been studied extensively, but the ectocyst is believed to comprise an acid-insoluble protein-containing material, with the endocyst containing approximately 33% cellulose (18, 32, 34). A greater understanding of the molecular and biochemical factors controlling the *Acanthamoeba* encystment process and the composition of the cyst wall is clearly required. This would also aid in the development of improved therapeutic and disinfectant agents in both the treatment and the prevention of *Acanthamoeba* keratitis.

This study has demonstrated some fundamental factors that can influence the observed efficacies of disinfectants and therapeutic agents against *Acanthamoeba* cysts. In view of the variety of experimental approaches and different outcomes, standardization for *Acanthamoeba* disinfectant efficacy testing, which is required for bacteria and fungi, is clearly needed (6, 11). This should take into account the *Acanthamoeba* species and strain used, the time in laboratory culture, the method of cyst preparation, and the cyst storage period. While encystment methods such as trophozoite culture in the presence of Mg^{2+} provides the large numbers of cysts that are usually required for molecular, biochemical, and differentiation analyses, the results obtained by their use in disinfection studies should be interpreted cautiously, as cysts are more susceptible to killing by biguanide and amidoamines.

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