Efficacy Of Regard® and Oxysept® 1 Step Against Acanthamoeba species: Regimen Testing

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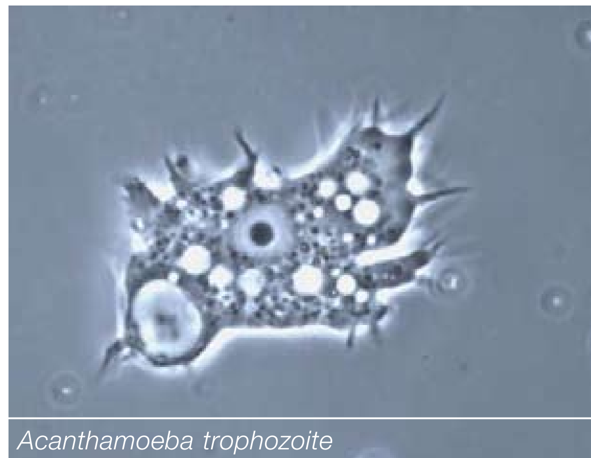
Introduction

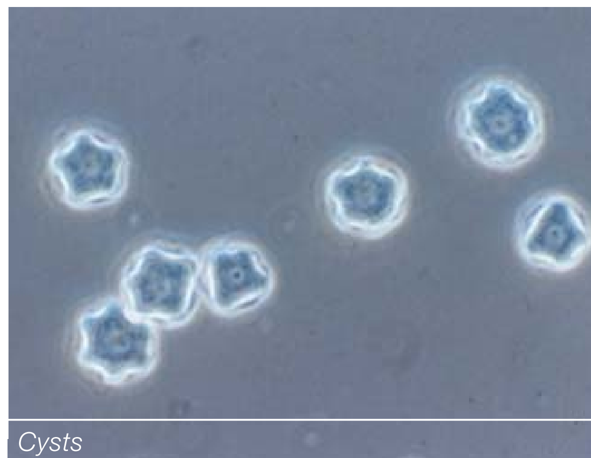
Acanthamoeba is a free-living amoeba characterised by a life cycle of feeding and replicating trophozoite and dormant cyst stage. The resistance of *Acanthamoeba* cysts to extremes of temperature, disinfection and desiccation accounts for the presence of the organism in virtually all soil and freshwater habitats.[5]

Acanthamoeba are pathogenic to humans, causing a potentially blinding infection of the cornea (keratitis). Contact lens wearers are most at risk from Acanthamoeba keratitis and account for some 90% or reported cases.[6] Poor hygiene practices such as failure to comply with recommended contact lens disinfection and cleaning procedures, and the rinsing or storing of lenses in tap water are recognised risk factors in acquiring the infection.[4]

In this study the efficacy of a novel contact lens multipurpose disinfectant solution (MPS: Regard®) and a one-step hydrogen peroxide system (Oxysept® 1-Step) were tested for their ability to remove *Acanthamoeba* spp. trophozoites and cysts from soft contact lenses.



Acanthamoeba trophozoite



Cysts

The disinfection action of Regard is the result of the production of chlorine dioxide by the activation of a complexed molecule composed of sodium chlorite and hydrogen peroxide. Chlorine dioxide is powerful oxidizing agent that penetrates the micro-organisms' membrane and destroys cellular components. A trace amount of H_2O_2 is employed to maintain the stability of the notoriously unstable chlorite ions. Previous studies have documented this molecule's effectiveness in destroying killing Gram + and Gram – bacteria, yeasts and fungi. Interestingly, the disinfectant is reduced to salt, water and oxygen in the ocular environment.

Materials and Methods

***Acanthamoeba* strains**. *A. polyphaga* (Ros) and *A. castellanii* (ATCC 30868) were maintained at 30°C in an axenic broth medium composed of Biosate (BBL: Becton Dickinson, UK) 20.0 g; glucose 5 g; KH_2PO_4 0.3 g; Vitamin B_{12} 10 µg; L-Methionine 15 mg per litre of de-ionised water. The pH was adjusted to 6.5-6.6 with 1 M NaOH before autoclaving at 121°C for 12 minutes.[3]

Cysts were prepared from trophozoite cultures grown in the axenic medium that had been supplemented with 50 mM $MgCl_2$.[2] The encystment medium was inoculated with approximately 2 x 10^5/ml trophozoites and incubated 30°C in a shak-

ing incubator (100 rpm) for 7 days. Cells were enumerated with a haemocytometer. Microscopic examination showed >90% mature cysts, which were stored at 4°C for testing within 14 days.[2]

Disinfectant solutions and contact lenses. The MPS Regard® and the one-step hydrogen peroxide system Oxysept® 1-Step were the test solutions. Phosphate buffered saline (PBS) served as the control.

The test lenses were new, unworn Frequency 55® (CooperVision) and Acuvue® Advance™ with Hydraclear™ (Johnson & Johnson).

Regimen testing (No Rub and Rinse protocol). The method for conducting the No Rub and Rinse testing is given in Appendix 1 Trophozoites or cysts were washed three times with PBS + 0.05% (weight/volume) Tween 80 and adjusted to 1×10^7/ml. Four separate 10 µl volumes of trophozoites or cysts (i.e. 1×10^5) were spaced in a petri dish. Four lenses were removed from their packets and placed convex side down onto the inocula. A second 10 µl volume of the amoebae was added to the concave side of the lenses and left to incubate in a humidified chamber at room temperature for 90 minutes.

Following incubation each lens was picked up with a sterile forceps and one side rinsed with a steady stream of Regard® for 5 seconds (approximately a total of 10 ml volume of the solution). The lens was then held with a fresh pair of sterile forceps and the other side rinsed with a second stream of solution for 5 seconds (Appendix 1). The lens was then placed into the well of a 12-place microtitre plate containing 3 ml of Regard® and left to soak for 4 hours.

With Oxysept® 1-Step, the inoculated lenses were disinfected according to the manufacturer's recommendation. Briefly, the inoculated lenses were placed into the lens storage case supplied with the system and containing 10 ml of the hydrogen peroxide (3% v/v) solution. A neutraliser tablet was then added and the case inverted three times and left for 6 hours.

Enumeration of viable *Acanthamoeba* on lenses. With the Regard® system, after the lenses had soaked for 4 hours, they were removed and placed into wells of a 24-place microtitre plate containing 1 ml of disinfectant neutraliser (0.05% Tween 80, 0.6% sodium thiosulphite and 0.02% catalase in 1⁄4 strength Ringer's solution). The lenses in the well were rinsed vigorously in situ with a pipette to detach any *Acanthamoeba* and serial 10-fold dilutions were made across the other wells (0.1 ml + 0.9 ml 1⁄4 strength Ringer's). Live *Escherichia coli* was added to each well (25 µl of approximately 5×10^7 bacteria /ml) and the microtitre plates incubated at 32°C for 7 days. In the presence of live *E. coli*, viable cysts excyst (hatch) and the emergent trophozoites feed and replicate (Appendix 2).

With Oxysept® 1-Step system, after the lenses had been soaked for 6 hours they were removed from the storage case and processed as described above for the Regard® study.

In control experiments, the inoculated lenses were processed as in the Regard® study except that PBS was used throughout.

The wells were inspected for *Acanthamoeba* growth after 48 h and then daily for up to 7 days. The number of surviving organisms on the lenses was calculated using Spearman-Karber computations from the presence or absence of excystment in the in the wells containing the serial 10-fold dilutions of the processed lenses, as described above.[1]

Each lens type was tested in quadruplet with each disinfectant or PBS control.

Results

Regimen testing (No Rub and Rinse). The findings for the regimen testing are shown in the Table.

Following the No Rub and Rinse protocol with the Regard® solution no surviving trophozoites or cysts of either *Acanthamoeba* spp. were recovered from any of the lens types, all tested in quadruplet (Table).

With the Oxysept® 1-Step system, no trophozoites were recovered from the lenses (Table). However, survivors were detected with the cysts (Table).
In the PBS control experiments trophozoites and cysts of both species were detected on the lenses (Table).

Table. Regimen testing (No Rub and Rinse) against *Acanthamoeba* spp.

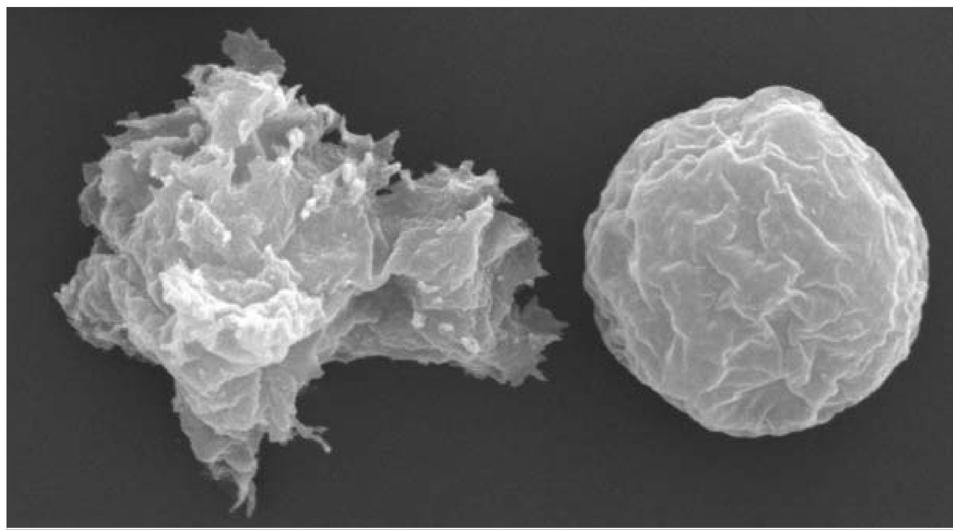
	A. polyphaga		*A. castellanii*	
Solution & Lens Type	Trophozoites	Cysts	Trophozoites	Cysts
Regard®				
Frequency 55®	<1*	<1	<1	<1
Acuvue® Advance™ with Hydraclear™	<1	<1	<1	<1
Oxysept® 1-Step				
Frequency 55®	<1	56	<1	100
Acuvue® Advance™ with Hydraclear™	<1	32	<1	32
Phosphate buffered saline				
Frequency 55®	112	18	63	92
Acuvue® Advance™ with Hydraclear™	162	97	92	51

*mean value from 4 separate lens experiments

Discussion

Under the No Rub & Rinse conditions described in this study, Regard® was found to be effective in removing *Acanthamoeba* spp. from soft contact lenses. As the contact lens is the vector by which *Acanthamoeba* become inoculated on to the cornea, the ability to remove the organism may help reduce the incidence of keratitis among this group.

Although the Oxysept® 1-Step showed no recovery of trophozoites, the cysts were detected on the lenses following disinfection with the system. However, it should be



EM troph and cyst

noted that the one-step peroxide system does not employ a rinse step prior to disinfection as was used with the Regard®. Therefore, the lenses containing the challenge inoculum of 2×10^5 *Acanthamoeba* trophozoites or cysts are added to the storage case containing the hydrogen peroxide solution. The presence of the neutralising tablet results an effervescent action from the decomposition of the peroxide and this may cause removal of adhering organisms from the lenses into the solution. Some of the solution containing the detached *Acanthamoeba* is recovered with the lenses when they are removed from the case for analysis. This feature makes it impossible to estimate accurately the removal of *Acanthamoeba* from the lenses in the Oxysept® 1-Step system.

In a previous study it has been shown that this one-step hydrogen peroxide system is effective in killing *Acanthamoeba* trophozoites but not cysts and so accounts for the absence of viable trophozoites on the lenses after the 6 hour disinfection cycle.[3]

Statement: I certify that all information contained in this document was obtained from the study conducted by myself, according to approved methods and procedures.

Date: 05 January 2005

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Reference List

1. Hamilton, M. A., R. C. a. Russo, and R. V. Thurston. 1977. Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. Environ. Sci. Technol. 11:714-719; Correction 12:417 (1978).
2. Hughes, R., W. Heaselgrave, and S. Kilvington. 2003. *Acanthamoeba polyphaga* strain age and method of cyst production influence the observed efficacy of therapeutic agents and contact lens disinfectants. Antimicrob Agents Chemother 47:3080-4.
3. Hughes, R. and S. Kilvington. 2001. Comparison of hydrogen peroxide contact lens disinfection systems and solutions against *Acanthamoeba polyphaga*. Antimicrobial Agents and Chemotherapy 45:2038-43.
4. Kilvington, S., T. Gray, J. Dart, N. Morlet, J. R. Beeching, D. G. Frazer, and M. Matheson. 2004. Acanthamoeba keratitis: the role of domestic tap water contamination in the United Kingdom. Invest Ophthalmol Vis Sci 45:165-9.
5. Kilvington, S. and D. G. White. 1994. *Acanthamoeba*: biology, ecology and human disease. Reviews in Medical Microbiology 5: 12-20.
6. Radford, C. F., D. C. Minassian, and J. K. Dart. 2002. Acanthamoeba keratitis in England and Wales: incidence, outcome, and risk factors. British Journal of Ophthalmology 86:536-42.

Appendix 1. No Rub & Rinse Protocol

1. Harvest trophozoites or cysts and wash x3 with PBS by centrifugation at 1000 x g for 5 minutes.

2. Resuspend pellet in PBS and adjust concentration to 1×10^7 /ml.

3. In a petri dish, pipette four 10 µl volumes of organism (i.e. 1×10^5 /ml)

4. Remove 4 lenses with forceps, blot on sterile gauze and place convex side down onto inocula.

5. Add a second 10 µl volume of organism to the concave side of the lenses.

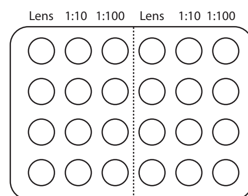
6. Leave for 90 minutes in humidified chamber then:

I. With forceps, pick up lens.
II. Rinse one side of lens with the appropriate solution for 5 seconds each.
III. Using a second pair of sterile forceps hold the lens and rinse the other side of lens with the appropriate solution for 5 seconds each.
IV. Place lens concave side up into well of a 12-place microtitre plate containing appropriate volume of test solution or control PBS.
V. Leave for 4 hours at room temperature.

7. Detect for adhering and viable surviving organisms as described in Appendix 2.

Appendix 2. Determining Lens Surviving Organisms

1. Divide plate as shown and add 1 ml of neutraliser to "Lens" wells and 1⁄4 strength Ringer's solution to remainder.



2. Transfer lenses to wells marked "Lens".

3. Rinse lenses thoroughly in the wells with a 1 ml pipette 6 times.

4. Make serial 10-fold dilutions across the remaining wells of the plate (i.e. remove 0.1 ml from "Lens" well and add to "1:10" row, mix 6 times and add 0.1 ml to "1:100" row.

5. Add 1 drop of *Escherichia coli* from a 3 ml pastette to each well (from a 1:20 dilution of the lab stock culture)

6. Incubate plate at 32°C for 7 days.

7. Inspected wells for *Acanthamoeba* growth after 48 hours and then daily for up to 7 days.

8. Score wells and calculate number of *Acanthamoeba* by Spearman-Karber computation.[1]

[1] Hamilton, M.A., R.C. Russo, and R.V. Thurston (1977). Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. Environ. Sci. Technol. 11: 714-719; Correction 12:417 (1978).

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