Reports

Quantitative Enumeration of Acanthamoeba for Evaluation of Cyst Inactivation in Contact Lens Care Solutions

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A simple, quantitative plate assay has been developed for use in the enumeration of Acanthamoeba. The technique uses an agarose overlay and low-nutrient medium to support the growth of amoebae on a bacterial lawn. The authors found that in this assay, individual trophozoites or dormant cysts will cause plaques to form in an Enterobacter aerogenes lawn. With the assay, it is possible to quantitatively assess the effects of various disinfectant compounds on the viability of Acanthamoeba. The authors used this assay to enumerate Acanthamoeba cyst viability after chemical disinfection in contact lens care solutions. The inactivation data indicated major differences among the four test solutions evaluated. Invest Ophthalmol Vis Sci 32:655–657, 1991

Infections caused by protozoa, such as Acanthamoeba keratitis, are difficult to treat because the organisms undergo a life cycle that involves encystation to resistant forms. Cysts can remain viable in airborne transmission due to their ability to withstand extremes of desiccation and temperature. They are also more resistant than trophozoites to nutrient starvation, salinity, and pH. Some cases of Acanthamoeba keratitis have been attributed to contaminated contact lenses, primary sources being aqueous solutions, as in the case of hot tubs or homemade saline solutions.1–4 Current clinical detection of Acanthamoeba is based on fluorescence and light microscopy. Although this method can be used for quantitative enumeration, it does not differentiate between viable and nonviable amoebae. Silvany et al5 described a qualitative technique that determines the viability of Acanthamoeba to evaluate the effectiveness of contact lens disinfectant solutions. The technique, although rapid, did not discriminate between cysts and trophozoites and did not avail itself to quantitative analysis. This report introduces a technique that can be used for quantitative enumeration of viable amoebae. In this technique, not only are the numbers of viable Acanthamoeba determined, but also differentiation between cysts and trophozoite sensitivity to disinfectants can be achieved. Trophozoites can be selectively killed by pretreatment of the samples at 55°C for 1 hr without a significant effect on cyst viability (T. J. Byers, personal communication, 1990). Inactivation studies require quantitative, simple methods for viable counting of protozoan trophozoites and cysts. Current contact lens disinfection systems are designed to inactivate bacteria; consequently, little experimental work has been published on quantitative testing of formulations of contact lens solutions effective against pathogenic protozoa. In this report, a simple method is presented that was adopted to evaluate Acanthamoeba cyst inactivation in various commercial formulations developed for contact lens care.

Materials and Methods. The organism used for this study was the Neff strain of Acanthamoeba castellani. This strain, which was originally isolated from a soil sample, has been maintained axenically for approximately 30 yr.6 The organism was maintained in a modification of Neff’s optimal growth medium (OGMA) in which the amount of yeast extract was reduced by 50% to limit precipitate formation.7 Amoebae were grown in disposable plastic tissue culture flasks. The OGMA was replaced with Neff’s encystment medium when the culture density reached approximately 1000 amoebae/mm² to induce synchronous encystment.7 The cultures that encysted were incubated for 5 days at 30°C. Mature cysts were harvested; the tissue culture flask was scraped with a sterile rubber policeman to dislodge adherent cysts. The cysts were then pelleted by centrifugation at 2000 × g for 10 min in a refrigerated centrifuge. The cysts were resuspended in 10 ml of 0.15 M KCl, sonicated for 30 sec in an ultrasonic cleansing bath, and then passed 20 times through a 21-gauge hypodermic needle. This treatment dispersed cysts and lysed immature cysts and trophozoites, as determined by microscopic examination. Only cysts with a thick double wall were scored as mature. Total cyst numbers were determined by hemacytometer counts. Cysts were pelleted a final time

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and resuspended at \(1 \times 10^5\) cysts/ml in 0.15 M KC1. The final suspension contained about 99% cysts. The cyst suspension could be stored at 4°C for at least six weeks with no detectable loss in cyst viability.

An agar sandwich technique that enumerates viable *Acanthamoeba* cysts was developed by Jensen and Dubes. In this study, a simpler method similar to one proposed by Angler et al was used, based on the use of a nutrient-limited agar/agarose overlay technique. Media used for this technique were developed for the growth of *Acanthamoeba* cysts. SM/5 agar was used as a low-nutrient base agar, and top agarose was a nutrient-limited overlay developed for the growth of lawns of enteric bacteria. To prepare feeder bacteria, a lawn of *Enterobacter aerogenes* was grown overnight on SM agar in a 100-mm petri dish. The lawn was washed off the plate with 3 ml of 0.15 M KC1 and stored on ice until used. Cysts (0.1 ml) were spread on the surface of an SM/5 plate with sterile glass spreader, and the plate was allowed to dry. A 75-μl aliquot of the *E. aerogenes* suspension was added to 2.4 ml of molten top agarose (held at 50°C). The suspension that resulted was poured onto the cyst containing SM/5 plate. Amoebae that re-excysted with plaque counts may also be caused by small air bubbles in the medium. These can be discriminated against the dense background. Interference with a sterile glass spreader, and the plate was allowed to dry. A cleared zone in the bacterial lawn. Plaques became visible within 3-5 days of incubation at 30°C. because plaques in the bacterial lawn were relatively large when they became distinctly clear, in most cases it was desirable to count plaques with 200 or fewer plaques per plate. In this assay, there was a one-to-one correlation between plaque number and viable cyst number. The plaque assay gave linear results over a two-log range of viable cysts per plate. If properly sealed, the plates could be stored for 2-3 months in cold without a loss of the characteristic plaque. At a low density of feeder bacteria, the plaques remained small and difficult to quantitate; if too high (\(>1.8 \times 10^9\) bacteria per plate), the plaques were difficult to discern against the dense background. Interference with plaque counts may also be caused by small air bubbles in the medium. These can be discriminated under a microscope and avoided by careful preparation of these plates.

Four chemical disinfectant solutions were tested:

- Solution A: Active ingredient 0.003% benzalkonium chloride. (Allergen Optical, Inc., Irvine, CA).
- Solution B: Active ingredient 0.0015% polyaminopropyl biguanide (PABP). (Polymer Technology Corp., Wilmington, MA).
- Solution C: Active ingredient 0.005% chlorhexidine gluconate. (Polymer).
- Solution D: Active ingredient 0.006% chlorhexidine gluconate. (Sola/Barnes-Hind, Sunnyvale, CA).

Solutions A, C, and D were purchased commercially. Solution B was provided by the manufacturer. At the time of the investigation, it was classified as an investigational product. Cysticidal activity was assayed as follows. Cysts (total number \(1 \times 10^4\)) were pelleted in a 1.5-ml microcentrifuge tube by centrifugation at 16,000 \(\times g\) for 1 min. The viability of the cysts was unaffected by this step. The cysts were re-suspended in 1 ml for the solution that was tested, and a 200-μl sample was immediately withdrawn. The first samples represent a 45-sec exposure to the test solution. The remainder of the cyst suspension was incubated at ambient temperature (23–26°C), and cysts were pelleted in the microcentrifuge to process samples at the time of collection. The cysts were washed once in 0.15 M KC1, and decimal dilutions were made of the washed cysts. Viable cysts in the samples were then determined by the standard plaque assay. All plaque assays were performed in triplicate.

**Results.** Disinfection results are summarized in Table 1. Two of the four solutions (A and B) yielded incomplete inactivation within 8 hr. Solution B, based on PABP formulation, yielded the lowest inactivation (two orders of magnitude decrease in viability in 8 hr). Viable cysts were not recovered after 4 hr of contact in the other two test solutions (C and D), both of which were based on chlorhexidine formulations. Solution D, which yielded the fastest inactivation, was also tested over a 2-hr time course. In this separate experiment, the viable cysts counts (per ml) decreased from \(1.5 \times 10^5\) to \(1.2 \times 10^5\) (45 sec), \(4.1 \times 10^3\) (30 min), \(1.9 \times 10^2\) (60 min), and \(7.8 \times 10^2\) (2 hr). Loss of viability over a similar time course was

<table>
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<tr>
<th>Contact time (h)</th>
<th>Viable cysts/ml*</th>
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<tr>
<td></td>
<td>Solution A</td>
</tr>
<tr>
<td>45 sec</td>
<td>(~1 \times 10^4)</td>
</tr>
<tr>
<td>2 hr</td>
<td>(1.5 \times 10^4)</td>
</tr>
<tr>
<td>4 hr</td>
<td>(2.1 \times 10^4)</td>
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<tr>
<td>8 hr</td>
<td>(8.5 \times 10^4)</td>
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* Results are the average of triplicate assays of one trial. The initial density was \(1.5 \times 10^5\) viable cysts/ml.

†† Viable counts were based on plates with < 300 plaques/plate.

†† Nd, not determined in this 8-hr time course experiment.
not apparent in control cysts that were not exposed to a disinfectant.

**Discussion.** Effective disinfection of *Acanthamoeba* is essential for safe lens wear. Clinical cases of *Acanthamoeba* keratitis in patients who wear rigid gas-permeable contact lenses, as well as the previously reported problems that originate from homemade saline solutions, underscore the importance of lens care formulations for effective cyst inactivation. The viable counting technique presented here is available to test lens care solutions in search of more effective chemical disinfection methods. The experimental data presented in this report show the applicability of this technique to produce quantitative data not accomplished by previously published methods.

**Key words:** *Acanthamoeba* enumeration, cyst inactivation, disinfection, rigid gas-permeable contact lenses, contact lens solutions

**Acknowledgments.** The authors thank Dr. Thomas J. Byers for advice and facilities, and Ms. Laurie Haldeman for typing the manuscript.

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