Development of a Practical Complete-Kill Assay to Evaluate Anti-Acanthamoeba Drugs

Regis P. Kowalski, MS, M(ASCP); Salwa Abdel Aziz, MD; Eric G. Romanowski, MS; Robert M. Q. Shanks, PhD; Amy C. Nau, DO; Leela V. Raju, MD

**IMPORTANCE** Acanthamoeba keratitis is a debilitating eye disease that requires effective topical drug therapy. Currently, there is no standard in vitro test to evaluate anti-Acanthamoeba drugs.

**OBJECTIVE** To develop a practical in vitro complete-kill assay to assess anti-Acanthamoeba drugs.

**DESIGN AND SETTING** Isolates of Acanthamoeba strains (n = 15) evaluated in a clinical laboratory. An in vitro laboratory assay was created to determine whether polyhexamethylene biguanide, 0.02%, chlorhexidine digluconate, 0.02%, hexamidine disethioonate, 0.1%, and voriconazole, 1.0%, were effective in completely killing 15 different isolates of Acanthamoeba at time points of 24, 48, and 72 hours in comparison with a saline control. Each 0.5-mL volume of drug was inoculated with 0.1 mL of Acanthamoeba cysts (range, 1 - 3 × 10⁶/mL) (determined with a hemacytometer) and allowed to incubate at 30°C. At the time points listed, 0.05 mL from each treatment group was inoculated onto nonnutrient agar overlaid with Enterobacter aerogenes. The plates were microscopically examined for growth 1 and 2 weeks after inoculation. At 2 weeks, all plates were subcultured onto a fresh medium. At another 7 days, the growth in subculture at each time point was graded “1” for growth and “0” for no growth.

**MAIN OUTCOMES AND MEASURES** The cumulative grades of 3 time points (range, 0-3) for each drug and isolate were nonparametrically compared to determine differences in growth between the drugs. The “kill” incidence rates over the 3 time points were also compared.

**RESULTS** In vitro testing determined that antiacanthamoebal efficacy (determined by the median growth grade and the kill incidence rate) was more prominent for hexamidine disethioonate (median growth grade, 0.0; kill incidence rate, 93% [14 of 15 isolates]) and polyhexamethylene biguanide (median growth grade, 0.0; kill incidence rate, 80% [12 of 15 isolates]) than for chlorhexidine digluconate (median growth grade, 1.0; kill incidence rate, 40% [6 of 15 isolates]), voriconazole (median growth grade, 2.0; kill incidence rate, 13% [2 of 15 isolates]), and saline (median growth grade, 3.0; kill incidence rate, 0% [0 of 15 isolates]).

**CONCLUSIONS AND RELEVANCE** The complete-kill assay appears to provide separation in the effectiveness of different antiamoebic drug solutions. This assay may be helpful for guiding topical Acanthamoeba therapy and providing a practical method to evaluate and screen new anti-infectives in the treatment of Acanthamoeba keratitis.
Acanthamoeba keratitis is a sight-threatening infection causing progressive corneal disease that can be difficult to treat and that can lead to corneal transplant. The infection is caused by free-living Acanthamoeba species, which are ubiquitous in the environment. Acanthamoeba keratitis is more prevalent among individuals who wear soft contact lenses, with an estimate of 1 to 2 cases per 1,000,000 contact lens wearers in the United States. An outbreak in the United States in 2007 has put more emphasis on the treatment of Acanthamoeba keratitis. Under adverse conditions, the amoebae encyst, and medical therapy is often less effective against cysts than against trophozoites. In recent years, several medications have been used for the treatment of Acanthamoeba keratitis; however, success in eradicating the organism varies widely. The reason for this variation is not clear and could be dependent on the individual species, its resistance to certain drugs, host immunity, or the effectiveness of the medication itself. Currently, there is no standard in vitro testing to guide therapy, and the decision is left to the clinician to choose from a variety of available medications. In addition, there is no practical in vitro test to screen new potential anti-Acanthamoeba compounds for therapy, or to screen contact solutions to ensure disinfection against Acanthamoeba contamination.

The primary goal of our study was to establish a practical in vitro test to evaluate the efficacy of 4 anti-Acanthamoeba drugs (polyhexamethylene biguanide [PHMB], 0.02%; chlorhexidine digluconate, 0.02%; voriconazole, 1.0%; and hexamidine diisethionate, 0.1%) against 15 Acanthamoeba isolates based on a 100% amoebicidal effect over a 3-day incubation period. It is expected that the steady state concentration of the drug with Acanthamoeba cysts would result in completely killing the Acanthamoeba species, thus indicating effective activity. If not, this would suggest that a drug may not be optimal for topical therapy, as in the treatment of keratitis.

Methods

Acanthamoeba Isolates

The in vitro susceptibility test was evaluated with 1 isolate of Acanthamoeba castellanii (strain 30010 purchased from American Type Culture Collection) and 14 de-identified Acanthamoeba strains isolated from the corneas of patients with Acanthamoeba keratitis. The isolates were maintained at 4°C to 6°C on nonnutrient agar standard (80-mm) plates (Difco Agar Noble, reference 214230; Becton, Dickinson and Company) overlaid with Enterobacter aerogenes. Fifteen isolates were tested to determine whether there was variability in drug testing.

Enterobacter aerogenes

Enterobacter aerogenes (strain 25028 purchased from American Type Culture Collection) was the food source for propagating Acanthamoeba in culture. This bacterium has been a mainstay for processing corneal specimens for Acanthamoeba growth in our clinical laboratory. The E aerogenes was grown for 24 hours on tryptase soy agar supplemented with 5% sheep red blood cells at 37°C. (Personal experience [R.P.K.] has determined that 24-hour growth was less likely to cause bacterial aggregates that can be mistakenly identified as Acanthamoeba.) The plate was inoculated as directed for disk diffusion susceptibility by streaking with a soft-tipped applicator for 3 passes over one-third of the plate. The inoculum was created from very turbid suspension (slurry) or from another agar plate. A slurry was produced by collecting one-half of the 24-hour E aerogenes growth and suspending it in 5 mL of saline (0.5% wt/vol) (API, bioMérieux).

Anti-Acanthamoeba Drugs

The in vitro testing was evaluated with 4 anti-Acanthamoeba drug preparations commonly used for the treatment of Acanthamoeba keratitis: (1) PHMB, 0.02% (Baquacil; Arch Chemicals Inc); (2) chlorhexidine digluconate, 0.02% (Sigma-Aldrich Co); (3) voriconazole, 1.0% (Leiter’s Compounding Pharmacy); and (4) hexamidine diisethionate, 0.1% (Désomédine; Bausch & Lomb and Leiter’s Compounding Pharmacy).

Anti-Acanthamoeba In Vitro Testing Protocol

For each Acanthamoeba isolate (inoculum), we used the following protocol: (1) A munificent sample of stock Acanthamoeba was inoculated on 2 nonnutrient agar plates with E aerogenes overlay by use of a soft-tipped applicator. (2) The 2 inoculated plates were allowed to incubate at 30°C for 7 days to accumulate Acanthamoeba cysts. (3) On day 7, Acanthamoeba cysts were harvested into 1.0 mL of saline by use of a soft-tipped applicator. The nonnutrient agar surface was swiped many times with a moistened soft-tipped applicator to reach a maximal concentration of Acanthamoeba cysts in the 1.0 mL of saline. (4) The concentration of Acanthamoeba cysts (cysts per milliliter) was determined with a microscope at ×20 magnification using a hemacytometer. The maximum concentration of Acanthamoeba cysts collected in this manner was approximately 1 to 3 × 10⁶ cysts/mL. (5) In vitro testing was performed at 3 time periods: 24, 48, and 72 hours.

24-Hour Testing

First, for each isolate, 0.1 mL of Acanthamoeba inoculum was pipetted into 0.5 mL each of PHMB, 0.02%; chlorhexidine digluconate, 0.02%; voriconazole, 1.0%; hexamidine diisethionate, 0.1%; and saline, respectively. Second, the inoculated drugs and saline solution were allowed to incubate at 37°C in an air incubator for 24 hours. Third, at 24 hours, 0.05 mL of the inoculated drugs and saline solution were plated on nonnutrient agar overlaid with E aerogenes using a glass rod to disperse the drug. This prevented a concentrated amount of drug to inhibit bacterial growth. The overlay was prepared by spreading 0.3 mL of the E aerogenes slurry on a nonnutrient agar with a soft-tipped applicator. The plates were incubated at 30°C in an air incubator. After another 24 hours, a second overlay of E aerogenes was administered to ensure the food source was available to the Acanthamoeba without any effect of residual drug. Fourth, all plates were monitored for the robust growth of Acanthamoeba resulting in a mixture of sparse trophozoites and predominant cysts at days 7 and 14. Robust growth at day 7 terminated testing with a positive result. Fifth, at day 14 (plates observed for the lack of robust growth), all plates were vigorously subcultured with a soft-tipped applicator onto fresh agar plates and retested.
nonnutrient agar overlaid with *E. aerogenes*. Sixth, after 7 days of incubation, all plates were monitored for the robust growth of *Acanthamoeba* resulting in a mixture of sparse trophozoites and predominant cysts. Finally, for each drug, positive growth was graded as a “4,” and no growth was denoted as “0.”

**48- and 72-Hour Testing**

Testing at 48 hours was the same as at 24 hours, except for the second step, whereas the inoculated drugs and saline solution were allowed to incubate at 37°C in an air incubator for 48 hours. Testing at 72 hours was the same as at 24 and 48 hours, except for the second step, whereas the inoculated drugs and saline solution were allowed to incubate at 37°C in an air incubator for 72 hours.

**Statistical Analysis**

The “graded growth data” was nonparametrically analyzed with the Mood median test and the Mann-Whitney test (Minitab). A cumulative grade was determined for the 3-day period for each drug and *Acanthamoeba* isolate. For example, if an *Acanthamoeba* isolate demonstrated growth at the 24-, 48-, and 72-hour time points, the total grade would be 3. Also, *Acanthamoeba* growth at the time points of 24 and 72 hours, but no growth at 48 hours, would be designated as a “2.” The grade range would be between 0 and 3. Each drug and the saline solution would have 15 cumulative grades representing 15 different *Acanthamoeba* isolates. A median was calculated for 15 cumulative grades. This analysis allows for paradoxical data, such as no growth at 24 hours but growth at either 48 or 72 hours. All data are included in the analysis.

The “kill” incidence rate of *Acanthamoeba* over each of the 3 time points was analyzed with the Fisher exact test (www.langsrud.com/fisher.htm). Any incidence of positive growth over the 3 time points was considered survival and not a kill. For example, *Acanthamoeba* survival at 24 hours, but not at 48 and 72 hours, was not considered an incidence of a kill. Negative growth at all 3 time points was denoted as a kill.

**Results**

Our Table details the descriptive statistics of 15 *Acanthamoeba* isolates’ survival after exposure to common anti-*Acanthamoeba* drugs. The nonparametric statistical analysis of graded growth data determined that the median growth scores of hexamidine diisethioonate (0.0), PHMB (0.0), and chlorhexidine digluconate (1.0) were less than those of voriconazole (2.0) and saline (3.0) (*P* = .001, determined by use of the Mood median test). The median growth score of hexamidine diisethioonate was equivalent to that of PHMB (*P* = .60, determined by use of the Fisher exact test) to that of hexamidine diisethioonate (93% [14 of 15 isolates]). The kill incidence rate of chlorhexidine digluconate (40% [6 of 15 isolates]) was less than that of hexamidine diisethioonate (*P* = .01, determined by use of the Fisher exact test) but equivalent to that of PHMB (*P* = .06, determined by use of the Fisher exact test). The kill incidence rate of chlorhexidine digluconate was equivalent (*P* = .21, determined by use of the Fisher exact test) to that of voriconazole (13% [2 of 15 isolates]) but greater (*P* = .02, determined by use of the Fisher exact test) than that of saline (0% [0 of 15 isolates]). The kill incidence rate of voriconazole was equivalent to that of saline (*P* = .48, determined by use of the Fisher exact test). The analysis of the kill incidence data supported the results of the analysis of the graded growth data.

**Discussion**

The foremost purpose of an in vitro susceptibility assay is to guide the antimicrobial therapy for infectious disease. The most efficient in vitro susceptibility testing would correlate directly with in vivo results. In general, antibacterial in vitro testing has been successfully correlated with in vivo outcome in the systemic treatment of bacterial infections. Limited correlation has been realized in the treatment of some fungal infections. There are no antimicrobial susceptibility standards for assessing topical therapy, but the systemic standards for assessing bacterial susceptibility to antibiotics can be used, if the assumption that the antibiotic concentrations in the ocular tissues are equal to or greater than the antibiotic concentrations in the serum. This assumption has worked well in the topical treatment of ocular bacterial infections.

As stated earlier, there is no standard in vitro susceptibility method to test anti-*Acanthamoeba* drugs. The ophthalmic field will need to rely on the tentative methods designed by us and presented in our study. The in vitro method is an extension of the routine *Acanthamoeba* culture used in clinical laboratory studies, which is practical and simple.

---

**Table. Descriptive Statistics of 15 Acanthamoeba Isolates After Exposure to Common Anti-Acanthamoeba Drugs**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Median Growth Grade</th>
<th>Kill Incidence Rate, (%) of Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (control)</td>
<td>3.0</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Polyhexamethylene biguanide, 0.02%</td>
<td>0.0</td>
<td>12 (80)</td>
</tr>
<tr>
<td>Chlorhexidine digluconate, 0.02%</td>
<td>1.0</td>
<td>6 (50)</td>
</tr>
<tr>
<td>Hexamidine diisethioonate, 0.1%</td>
<td>0.0</td>
<td>14 (93)</td>
</tr>
<tr>
<td>Voriconazole, 1.0%</td>
<td>2.0</td>
<td>2 (13)</td>
</tr>
</tbody>
</table>

\(a\) The median value of the growth grade of 15 *Acanthamoeba* isolates based on the cumulative score of 3 time points.

\(b\) Determined over 3 time points; any incidence of positive growth over the 3 time points was considered survival and not a kill. Negative growth at all 3 time points was denoted as a kill.

---

The kill incidence rate of PHMB (80% [12 of 15 isolates]) was equivalent (*P* = .60, determined by use of the Fisher exact test) to that of hexamidine diisethioonate (93% [14 of 15 isolates]). The kill incidence rate of chlorhexidine digluconate (40% [6 of 15 isolates]) was less than that of hexamidine diisethioonate (*P* = .01, determined by use of the Fisher exact test) but equivalent to that of PHMB (*P* = .06, determined by use of the Fisher exact test). The kill incidence rate of chlorhexidine digluconate was equivalent (*P* = .21, determined by use of the Fisher exact test) to that of voriconazole (13% [2 of 15 isolates]) but greater (*P* = .02, determined by use of the Fisher exact test) than that of saline (0% [0 of 15 isolates]). The kill incidence rate of voriconazole was equivalent to that of saline (*P* = .48, determined by use of the Fisher exact test). The analysis of the kill incidence data supported the results of the analysis of the graded growth data.
Development of a Practical Complete-Kill Assay

assay was based on the total kill of *Acanthamoeba* cysts. As recommended by the US Food and Drug Administration, our in vitro susceptibility assay was tested with a multiple number of *Acanthamoeba* isolates that were propagated in bacteria and allowed to starve for cyst formation.\textsuperscript{10} Our testing strain of bacteria was *E. aerogenes*, which we favored over *Escherichia coli*. Creating a consistent *E. aerogenes* and *Acanthamoeba* inoculum was no problem. Saline was an appropriate positive growth control, and PHMB demonstrated amoebicidal activity and was inexpensive as an effective treatment control. With nonparametric analysis, we were able to demonstrate a separation of activity between the saline solution (ie, the control) and the test drugs.

At this time, our method is tentative and open to possible refinement. As in other methods,\textsuperscript{15-18} the in vitro data cannot be correlated with the critical management of acute *Acanthamoeba* keratitis, which relies on other factors such as drug penetration, dosing schedule, innate immunity, virulence factors, adjunctive measures, and patient compliance. Our main assumption is that if *Acanthamoeba* survive a steady state direct contact with a drug for 72 hours, then the drug probably is not optimal for topical therapy. Our data demonstrate a need for an animal model to test topical drugs that penetrate the cornea and kill *Acanthamoeba* organisms.

In conclusion, we developed an in vitro susceptibility test, based on 100% kill incidence rate, to test the efficacy of anti-*Acanthamoeba* drugs to eradicate *Acanthamoeba* cysts. Our in vitro testing determined that antiacanthamoebal efficacy was more prominent for hexamidine disethionate and PHMB than for chlorhexidine digluconate and voriconazole. Our data differ from data by others who found chlorhexidine digluconate to be more effective.\textsuperscript{18} Testing with our assay may have had a more stringent end point with the demonstration of a 100% kill incidence rate. Our method represents an unmet need to test anti-*Acanthamoeba* drugs for therapy, to screen new drugs, and to test contact lens solutions.

**ARTICLE INFORMATION**


Author Contributions: Dr Kowalski had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Kowalski, Abdel Aziz, Romanowski, Shanks.

Acquisition of data: Kowalski, Abdel Aziz.

Analysis and interpretation of data: Kowalski, Raju.

Drafting of the manuscript: Kowalski, Abdel Aziz.

Critical revision of the manuscript for important intellectual content: Kowalski, Romanowski, Shanks, Nau, Raju.

Statistical analysis: Kowalski.

Obtained funding: Kowalski.

Administrative, technical, or material support: All authors.

Study supervision: Kowalski.

Conflict of Interest Disclosures: Drs Kowalski, Romanowski, and Shanks have been paid fees by Bausch & Lomb, Tampa, Florida, as specialty advisors for other nonrelated endeavors. No other disclosures were reported.

Funding/Support: We are grateful to the Pennsylvania Lions Club, the Charles T. Campbell Foundation, the Eye and Ear Foundation of Pittsburgh, Pennsylvania, the National Institutes of Health (core grant P30 EY008098), and the Research to Prevent Blindness, New York, New York, for their continued financial support. Dr Shanks has received a career development award from the Research to Prevent Blindness and a grant from the National Institutes of Health (AI085570).

Role of the Sponsor: The funding agencies had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; and preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

**REFERENCES**


