Development of Colorimetric Microtiter Plate Assay for Assessment of Antimicrobials against *Acanthamoeba*

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We have developed and optimized a 96-well microtiter plate assay, based on the reduction of alamarBlue, to assess the efficacies of much needed new antimicrobials against *Acanthamoeba* species. This assay has been optimized for determination of drug efficacy against two potentially pathogenic species, *Acanthamoeba castellanii* and *Acanthamoeba polyphaga*, and has been validated by comparison of their relative susceptibilities to chlorhexidine, a drug widely used to treat *Acanthamoeba* keratitis. The results demonstrate that the assay is comparable to a manual counting assay and that *A. polyphaga* is more resistant to chlorhexidine than *A. castellanii*. Thus, by use of the manual counting assay, 3.125 μM chlorhexidine was almost completely effective against *A. castellanii*, whereas this concentration was less than 20% effective against *A. polyphaga*. Similar results were obtained by the alamarBlue assay. The new assay was used to determine the relative susceptibilities of *A. castellanii* and *A. polyphaga* to the alkylphosphocholines (APCs) hexadecylphosphocholine (hexadecyl-PC; miltefosine) and octadecylphosphocholine (octadecyl-PC) as well as an alkylglycerolphosphocholine, edelfosine. Both APCs studied were equally effective against *A. castellanii*, but octadecyl-PC was less effective than hexadecyl-PC against *A. polyphaga*. Both APCs were more effective than edelfosine against both *Acanthamoeba* species. *A. polyphaga* was found to be significantly less susceptible to each of the phosphocholine analogues. The newly described assay offers a number of advantages over those described previously. It is less labor-intensive than previously described assays and is sensitive and rapid, and the results can be read in a nonsubjective manner. As it is based on a standard 96-well, microtiter plate, it is amenable to automation and high throughput.

Acanthamoeba species are predominantly free-living protozoa found ubiquitously throughout the environment. *Acanthamoeba* species are now recognized as the cause of *Acanthamoeba* keratitis and granulomatous *Acanthamoeba* encephalitis (GAE) in humans (7, 11). *Acanthamoeba* keratitis is an increasingly common and severe corneal infection. It is closely associated with contact lens wear and can affect immunocompetent individuals (2, 17, 20). In contrast, GAE is a disease of immunocompromised individuals, including those with AIDS, in whom it is invariably fatal.

Present therapeutic regimens for *Acanthamoeba* keratitis rely on topical applications of antimicrobials, including a combination of propamidine isethionate and neomycin or chlorhexidine. The need for these drugs to be applied every 15 to 60 min for a period of weeks makes treatment arduous. Corneal transplantation is often necessary due to the extensive damage caused by the parasites. Moreover, as present treatments are poorly effective against the cystic stages of the parasite, residual infection often remains following treatment and can even result in infection of transplanted corneas (18). No effective antimicrobial treatment for GAE has been described, although such therapy has been used with apparent effect as an adjunct to surgery (16, 19). This exemplifies the urgent need

for new and effective antimicrobials. A group of compounds that shows some promise for the treatment of *Acanthamoeba* infection are the phospholipid analogues alkylphosphocholines (APCs) (10, 22). These were originally designed as anticancer agents but have recently proven extremely effective for the treatment of leishmaniasis, prompting studies with a number of parasites, including *Trypanosoma cruzi*, *Entamoeba histolytica*, and *Acanthamoeba*, against which they demonstrate some efficacy (10). A further class of phospholipid analogues, the alkylglycerolphospocholines (AGPCs), has also shown some efficacy as antineoplastic and antiparasitic agents (10).

A major obstacle in the discovery and development of new inhibitors is the lack of a microtiter plate assay suitable for testing their efficiencies against axenic *Acanthamoeba* and applicable for high throughput. The assays used at present to determine the efficiency of potential *Acanthamoeba* inhibitors include manual counting with a hemocytometer (9) or staining with fluorescent viability dyes and flow cytometric analysis (14, 5). Other quantitative methods include the standard plaque assay (14, 13) and a quantitative microtiter method for the enumeration of track-forming units (6). The number of viable organisms has also been estimated by most probable number enumeration methods (4). An ideal assay would measure only live parasites, the results would be read in a nonsubjective manner, and the assay would not rely on manual counting. Furthermore, if it were based on a standard microtiter plate, it would be amenable to scaling for high-throughput analysis.

The alamarBlue assay has been used to quantitatively evaluate the proliferation of mammalian cell lines (1, 24), fungi

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(21, 23), and bacteria (3, 8, 12). The assay measures innate cellular metabolic activity, which reduces the alamarBlue dye and changes its color as a measurable indicator of the amount of viable cells that are present in a test sample. Specifically, alamarBlue is reduced by NADPH, reduced flavin adenine dinucleotide, reduced flavin mononucleotide, and the cytochromes produced inside the cells.

Here we show that the alamarBlue assay can be designed to measure quantitatively the proliferation and viability of *Acanthamoeba* trophozoites. We determined the relative cytotoxicity of chlorhexidine to *Acanthamoeba castellani* and *Acanthamoeba polyphaga* and demonstrate that the results of the alamarBlue assay and the manual counting assay are comparable. Furthermore, we determined the susceptibilities of both *Acanthamoeba* species to the AGPC edelfosine in comparison to those to the APCs hexadecylphosphocholine (hexadecyl-PC) and octadecylphosphocholine (octadecyl-PC).

MATERIALS AND METHODS

***A. castellanii* and *A. polyphaga*.** *A. polyphaga* (strain 1501/18) was obtained from Culture Collection of Algae and Protozoa (Cumbria, United Kingdom). *A. castellanii* was kindly obtained from Keith Vickerman (Glasgow, United Kingdom). *A. castellanii* and *A. polyphaga* trophozoites were grown in medium containing 20% mycological peptone (Sigma, Poole, United Kingdom) and 0.9% maltose (Sigma, Poole, United Kingdom) and supplemented with 1% penicillin, streptomycin, and amphotericin B (all from Sigma). The *Acanthamoeba* spp. were incubated at room temperature in 75-cm^2 tissue culture flasks until they reached 90 to 95% confluence and were then harvested or subcultured by using Accutase enzyme cell detachment medium (Sigma).

Determination of optimal culture volume for assays. *A. castellanii* and *A. polyphaga* cells were seeded in triplicate on a 96-well tissue culture plate with 100 μl of serial dilutions from a stock solution with 8×10^5 cells per ml to generate a calibration curve. Further wells were filled only with 100 μl of medium for use as a blank. This plating protocol was repeated by the addition of a further 100 μl of medium to all wells to allow comparison of the effects of medium volumes of 100 and 200 μl on metabolic activity and, thus, assay sensitivity. The alamarBlue assay reagent (Biosource, Europe, Nivelles, Belgium) was placed into each test well, which contained medium and cells at 100 or 200 μl, at an amount equal to 10% of the medium volume. Test plates containing the alamarBlue reagent were then incubated for 6 h. The well contents were assayed for alamarBlue reduction by measuring the absorbance of the wells at 570 and 600 nm on a Spectromax 190 plate reader (Molecular Devices, Wokingham, United Kingdom).

The percent reduction of alamarBlue was calculated by the formula

$$(\{[(\varepsilon ox\ \lambda_2)(A\ \lambda_1)] - [(\varepsilon ox\ \lambda_1)(A\ \lambda_2)]\}/\{[(\varepsilon red\ \lambda_1)(A'\ \lambda_2)]$$
$$- [(\varepsilon red\ \lambda_2)(A'\ \lambda_1)]\}) \times 100$$

where εred λ_1 is 155,677 (molar extinction coefficient of reduced alamarBlue at 570 nm); εred λ_2 is 14,652 (molar extinction coefficient of reduced alamarBlue at 600 nm); εox λ_1 is 80,586 (molar extinction coefficient of oxidized alamarBlue at 570 nm); εox λ_2 is 117,216 (molar extinction coefficient of oxidized alamarBlue at 600 nm); $A\ \lambda_1$ is the absorbance of the test wells at 570 nm; $A\ \lambda_2$ is the absorbance of the test wells at 600 nm; $A'\ \lambda_1$ is the absorbance at 570 nm of the negative control wells, which contained medium and alamarBlue but to which *Acanthamoeba* was not added; and $A\ \lambda'_2$ is the absorbance at 600 nm of the negative control wells, which contained medium and alamarBlue but to which *Acanthamoeba* was not added. The results are expressed as the mean for each triplicate culture ± standard error (SE).

Determination of optimal seeding densities of *Acanthamoeba*. *A. castellanii* and *A. polyphaga* cells were grown in 75-cm^2 flasks until they reached 90 to 95% confluence and were then harvested by using Accutase (Sigma). A stock of 10^6 cells per ml was produced for each organism. The cells were seeded in triplicate on the 96-well plate with 100 μl of serial dilutions of the stock to generate a calibration curve. Further wells were filled only with 100 μl of medium and were used as blanks. This cell standard curve protocol was repeated on four plates to determine alamarBlue reagent reduction after 24, 48, 72, and 96 h of cell incubation under normal culture conditions. At 6 h prior to the end of incubation, 10 μl of alamarBlue reagent was added to each test well, and the test plate

was incubated for 6 h at room temperature in the dark. The absorbance of each well was read at 570 and 600 nm by using a Spectromax 190 plate reader (Molecular Devices).

The percent reduction of alamarBlue was calculated by the formula

$$(\{[(\varepsilon ox\ \lambda_2)(A\ \lambda_1)] - [(\varepsilon ox\ \lambda_1)(A\ \lambda_2)]\}/\{[(\varepsilon red\ \lambda_1)(A'\ \lambda_2)]$$
$$- [(\varepsilon red\ \lambda_2)(A'\ \lambda_1)]\}) \times 100$$

where the constants and variables are as defined above. The results are expressed as the mean for each triplicate culture ± SE.

Comparison of the relative susceptibilities of *A. polyphaga* and *A. castellanii* to chlorhexidine, as assessed by the alamarBlue assay and manual counting methods. *A. castellanii* and *A. polyphaga* cells were grown in 75-cm^2 flasks until they reached 90 to 95% confluence and were harvested by using Accutase (Sigma). *A. castellanii* and *A. polyphaga* cells were seeded in triplicate at 1,250 cells per well in 50 μl of medium in 96-well tissue culture plates (Greiner, Bio-One, United Kingdom) or 5,000 cells per well in 200 μl of medium in 24-well tissue culture plates (Greiner) and were allowed to adhere for 3 h. Medium containing chlorhexidine (Sigma) at a concentration range of 100 to 1.56 μM was freshly prepared to volumes of 50 and 200 μl and was added to the wells containing cells. The test plates were incubated for 96 h under normal culture conditions. At 6 h prior to the end of incubation, 10 μl of alamarBlue reagent was added to each of the test wells of the 96-well plate. The test plates were incubated for 6 h at room temperature in the dark. AlamarBlue reduction was assessed as described above. The percent inhibition of alamarBlue reduction was calculated by the formula

$$\{[(\varepsilon ox)\lambda_2 A\ \lambda_1] - [(\varepsilon ox)\lambda_1 A\ \lambda_2 \text{ of treated } Acanthamoeba]/[(\varepsilon ox)\lambda_2 A^o\lambda_1]$$
$$- [(\varepsilon ox)\lambda_1 A^o\lambda_2 \text{ of untreated } Acanthamoeba]\} \times 100$$

where the constants and variables are as defined above, $A^o\lambda_1$ is the absorbance of the untreated control with alamarBlue at 570 nm, and $A^o\lambda_2$ is the absorbance of the untreated control with alamarBlue at 600 nm.

This number was subtracted from 100 to give the percent alamarBlue reduction relative to that for the untreated control cultures. The results are expressed as the mean for each triplicate culture ± SE.

For manual counting, *Acanthamoeba* cells treated with chlorhexidine in 24-well tissue culture plates were detached by repeated pipetting, and 100 μl of the cell suspension was removed from each well and mixed with 100 μl of trypan blue vital stain (Sigma). Each treatment was done in triplicate test wells. Differential viable and nonviable cell counts were performed for each test well by counting the cells in a hemocytometer at ×100 magnification under a light microscope. The results are expressed as the mean for each triplicate culture ± SE.

Susceptibilities of *A. castellanii* and *A. polyphaga* to hexadecyl-PC, octadecyl-PC and edelfosine by alamarBlue assay. The effects of hexadecyl-PC, octadecyl-PC, and edelfosine (Alexis Biochemicals, Nottingham, United Kingdom) on each species of *Acanthamoeba* were determined precisely as described above for chlorhexidine over 96 h. Each of the phospholipid analogues was initially dissolved in ethanol and diluted so that the final concentration of ethanol was less than 0.5% at the highest concentration used. For an ethanol control assayed in parallel, this or a lower concentration of ethanol had no significant effect on parasite multiplication, as assessed by alamarBlue reduction.

Statistics and experimental design. All experiments were performed at least twice, with similar findings each time. Comparison of the susceptibilities of *A. castellanii* and *A. polyphaga* to chlorhexidine, hexadecyl-PC, octadecyl-PC, and edelfosine were performed by the Mann-Whitney U test. Regression analyses were performed with Cricket Graph software (Computer Associates International, Islandia, N.Y.).

RESULTS

Determination of optimal culture volume for assays. For both *Acanthamoeba* species, metabolic activity measured over a 6-h period was higher when they were cultured in 100 μl of medium (Fig. 1a and b). When alamarBlue was incubated in 200 μl of culture medium at a concentration of 5.0×10^4 cells/well, approximately 53 and 60% of the alamarBlue was reduced by *A. castellanii* and *A. polyphaga*, respectively. In contrast, the alamarBlue reduction by *A. castellanii* and *A. polyphaga* incubated in 100 μl of culture medium at this cell concentration measured approximately 95 and 88%, respec-
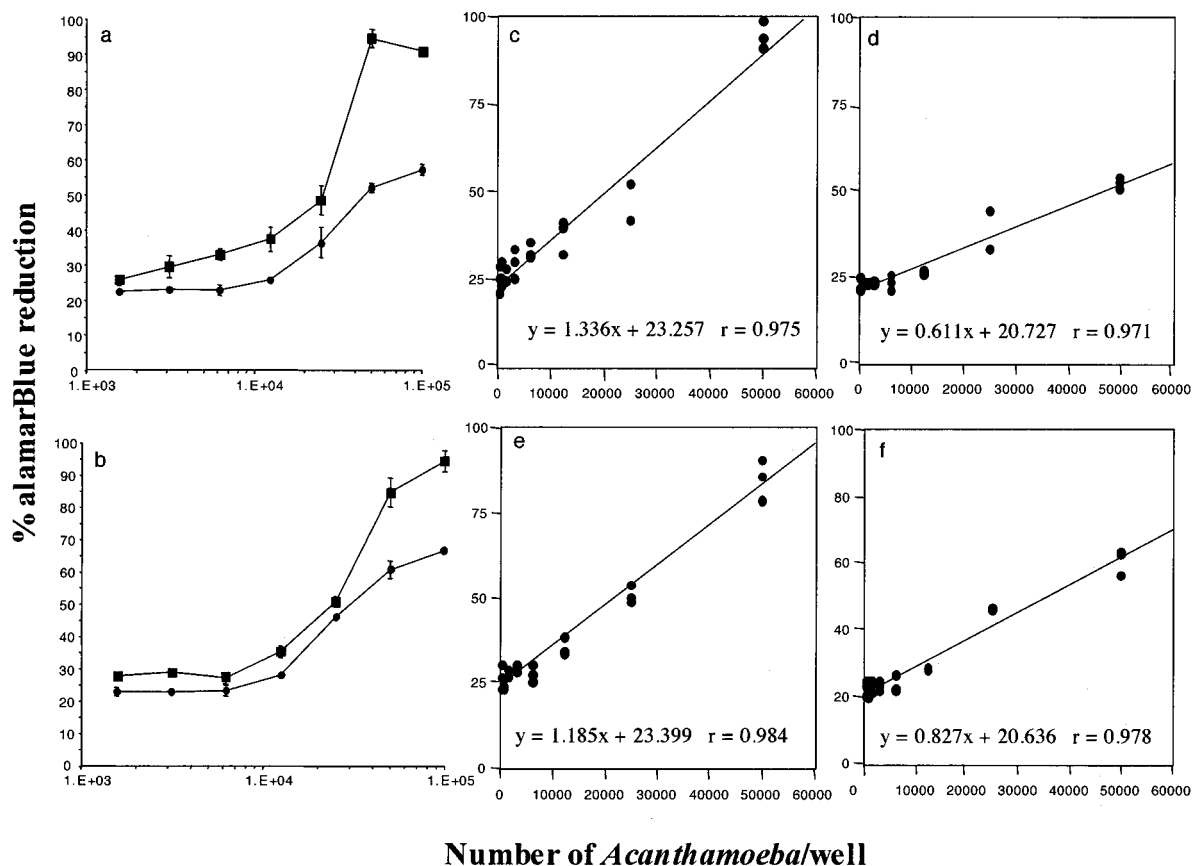
Number of *Acanthamoeba*/well

FIG. 1. Reduction of alamarBlue by different plating densities of *A. castellanii* (a) and *A. polyphaga* (b) cultures in 100 μl (closed squares) and 200 μl (closed circles) of medium over 6 h. In the 100-μl cultures, 95 and 88% alamarBlue reductions were achieved over this time period by 5.0 × 10⁴ *A. castellani* and *A. polyphaga* cells per well, respectively. In comparison, with the 200-μl cultures, maximum reductions of 53 and 60% alamarBlue were achieved by 5.0 × 10⁴ *A. castellani* and *A. polyphaga* cells per well, respectively. The results are expressed as the means for triplicate cultures ± SEs. (c to f) AlamarBlue reduction had a linear correlation with *Acanthamoeba* cell number, irrespective of the species or the culture volume, up to 5 × 10⁴ parasites per well ($P < 0.05$).

tively. At 1.56×10^3 cells well, the lowest concentration of *Acanthamoeba* plated, the alamarBlue reduction was between 20 and 30% for both species in both culture volumes. This indicates that a culture volume of 100 μl provides a larger dynamic range of alamarBlue reduction and thus allows the detection of small differences in *Acanthamoeba* cell number and effectively increases the resolution of the assay. This culture volume was used in all further studies.

Regression analyses of the data obtained for *A. castellanii* and *A. polyphaga* cultured in both 100- and 200-μl volumes demonstrated that the parasite number correlated with percent alamarBlue reduction in a linear manner up to 5×10^4 parasites per well ($P < 0.001$ for both *A. castellanii* and *A. polyphaga* at both culture volumes) (Fig. 1c to f).

Determination of optimal seeding densities for *Acanthamoeba*. The seeding densities of *A. castellanii* and *A. polyphaga* that attained close to 100% alamarBlue reduction were determined in assays conducted for total periods of 24, 48, 72, and 96 h. As anticipated for any given seeding concentration, the percentage of alamarBlue reduced increased with the length of incubation and correlated with the parasite number due to parasite proliferation (Fig. 2a and b). The optimum seeding densities for each time period, i.e., the density that

reduced the maximum levels of alamarBlue before the curves plateaued, were 1.25×10^3, 1.0×10^4, 2.0×10^4, and 8.0×10^4 at 96, 72, 48, and 24 h, respectively, for both *A. castellanii* and *A. polyphaga* (Fig. 2a and b). The remainder of the studies reported were carried out over 96 h and thus used 1.25×10^3 cells of both *A. castellanii* and *A. polyphaga*.

Comparison of the relative susceptibilities of *A. polyphaga* and *A. castellanii* to chlorhexidine, as assessed by the alamarBlue assay and manual counting methods. The effects of chlorhexidine on *A. castallanii* and *A. polyphaga* were consistent between the alamarBlue assay and the manual counting method, with the percent reduction of alamarBlue correlating with the *Acanthamoeba* parasite numbers (Fig. 3). Regression analyses of the data obtained for both *A. castellanii* and *A. polyphaga* demonstrated that live parasite numbers (the numbers of parasites except those that were stained with trypan blue) assessed by manual counting correlated with the percent alamarBlue reduction in a linear manner for both *A. castellanii* ($y = 13,090 + 26,827$; $R = 0.985$; $P < 0.001$) and *A. polyphaga* ($y = 5,685 - 4,004$; $R = 0.981$; $P < 0.001$) (results not shown).

A. castellanii was significantly more susceptible to chlorhexidine than *A. polyphaga*. Thus, complete inhibition of *A. castellanii* was achieved by 3.125 μM chlorhexidine, whereas this
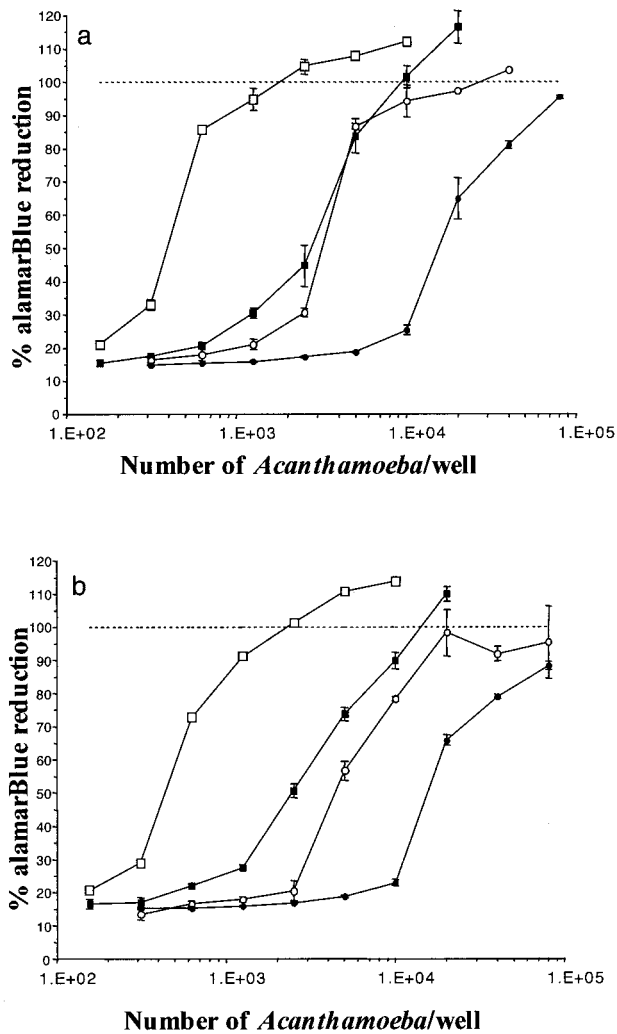
FIG. 2. Reduction of alamarBlue by different plating densities of *A. castellanii* (a) and *A. polyphaga* (b) cultured in 100 μl of medium for 96 h (open squares), 72 h (closed squares), 48 h (open circles), and 24 h (closed circles) prior to the addition of alamarBlue. The optimum plating densities, i.e., those that gave the highest alamarBlue reduction before the plateau, were determined to be 1.25×10^3, 1.0×10^4, 2.0×10^4, and 8.0×10^4 for 96, 72, 48, and 24 h, respectively, for both *A. castellani* and *A. polyphaga*. The results are expressed as the means for triplicate cultures ± SEs.
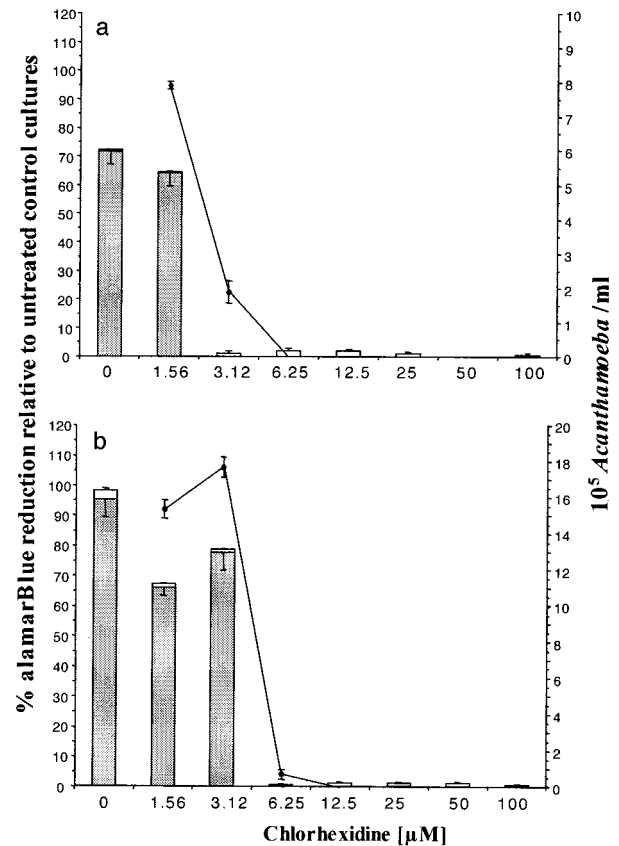

FIG. 3. Comparison of alamarBlue assay with manual counting assay for determination of the relative susceptibilities of *A. castellanii* (a) and *A. polyphaga* (b) to chlorhexidine over 96 h. Live cells (grey bars) and dead cells (white bars) were determined by trypan blue staining. The effect of chlorhexidine was measured in parallel cultures by determining the percent alamarBlue reduction relative to that for the untreated control cultures (closed circles). Note the comparable results obtained by both methods, which showed a linear correlation for both *A. castellanii* and *A. polyphaga* ($P < 0.05$). The results are expressed as the means for triplicate cultures ± SEs.

concentration of chlorhexidine inhibited multiplication of less than 20% of the *A. polyphaga* parasites ($P < 0.05$ by using data derived from both the alamarBlue assay and the manual counting assay). A chlorhexidine concentration of 6.25 μM was, however, completely effective (the 100% inhibitory concentration [IC_{100}]) against *A. polyphaga*. Irrespective of the assay used, IC_{50}s were between 1.5625 and 3.125 μM for *A. castellanii* and 3.125 and 6.25 μM for *A. polyphaga*.

Susceptibilities of *A. castellanii* and *A. polyphaga* to hexadecyl-PC, octadecyl-PC, and edelfosine by alamarBlue assay. *A. castellanii* was found to be significantly ($P < 0.05$) more susceptible to octadecyl-PC than *A. polyphaga* at all concentrations used up to 15.63 μM, which was completely effective against *A. castellanii* but which was only approximately 20% effective against *A. polyphaga*. IC_{50}s were between 3.906 and

7.813 μM for *A. castellanii* and between 31.25 and 62.5 μM for *A. polyphaga* (Fig. 4a). A similar pattern of relative susceptibility to hexadecyl-PC was observed, with *A. castellanii* being significantly ($P < 0.05$) more susceptible than *A. polyphaga* at all concentrations used up to 15.63 μM, which was almost completely effective against *A. castellanii* but which was only approximately 35% effective against *A. polyphaga*. IC_{50}s were between 3.906 and 7.813 μM for *A. castellanii* and between 15.63 and 31.25 μM for *A. polyphaga* (Fig. 4b).

Both *A. castellanii* and *A. polyphaga* were significantly ($P < 0.05$) more resistant to edelfosine than either hexadecyl-PC or octadecyl-PC at all concentrations used, with little effect observed below 250 μM (Fig. 4c). *A. castellanii* was significantly ($P < 0.05$) more susceptible than *A. polyphaga* to edelfosine at a concentration of 500 μM.

DISCUSSION

Species of the free-living protozoon *Acanthamoeba* are the causative agents of both *Acanthamoeba* keratitis and GAE in
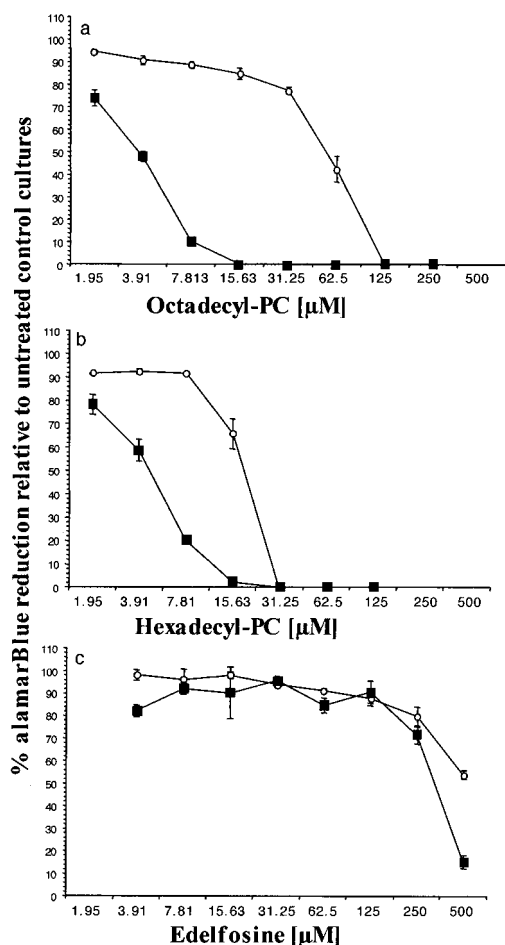
FIG. 4. Relative susceptibilities of *A. castellanii* (closed squares) and *A. polyphaga* (open circles) to the following over 96 h: (a) octadecyl-PC (IC$_{50}$s were between 3.906 and 7.813 μM for *A. castellanii* and between 31.25 and 62.5 μM for *A. polyphaga*); (b) hexadecyl-PC (IC$_{50}$s were between 3.906 and 7.813 μM for *A. castellanii* and between 15.63 and 31.25 μM for *A. polyphaga*); and (c) eldefosine (IC$_{50}$s were between 250 and 500 μM for *A. castellanii* and in excess of 500 μM for *A. polyphaga*). *A. castellanii* was significantly more susceptible than *A. polyphaga* to each of the phospholipid analogues tested ($P < 0.05$). *Acanthamoeba* cell numbers were assessed by measuring the percent alamarBlue reduction relative to that for the untreated control cultures over 96 h. The results are expressed as the means for triplicate cultures $\pm$ SEs.

humans (7, 19, 20). At present, there is an urgent need for new, effective antimicrobials (18). To facilitate this challenge we have developed a colorimetric microtiter plate assay that uses the reduction of alamarBlue to assess *Acanthamoeba* numbers and thus determine the efficacies of potential antimicrobial agents. This assay is sensitive, the results can be read in a nonsubjective manner, and the assay is less labor-intensive than existing assays. This assay system has been optimized for *A. castellanii* and *A. polyphaga*, the two most common species isolated from patients with *Acanthamoeba* keratitis.

The results of initial studies with a standard 200-μl culture volume were disappointing, as alamarBlue reduction reached a plateau at approximately 60%. This prompted studies to determine if an increase in the surface area-to-volume ratio by reduction of the culture volume would increase metabolic activity and result in greater alamarBlue reduction. These studies established that the culture volume did influence the metabolic activities of both species of *Acanthmoeba*, as measured by their abilities to reduce alamarBlue. Thus, by culturing *Acanthamoeba* in a volume of 100 μl and not one of 200 μl, alamarBlue reduction can reach essentially 100%, effectively increasing the assay resolution by allowing the detection of small differences in cell numbers. The larger surface area-to-volume ratio presumably serves to allow better oxygenation of the culture medium, which increases the metabolic activity of the *Acanthamoeba* cells and which results in increased production of the factors responsible for alamarBlue reduction (NADH, reduced flavin adenine dinucleotide, reduced flavin mononucleotide). An additional benefit of smaller volumes of culture medium is the economy of reagents. By using a titration of each *Acanthamoeba* species, the optimal seeding densities, i.e., those that gave maximum alamarBlue reduction during the exponential phase, were easily determined for a number of assay lengths ranging from 1 to 4 days.

By using the established parameters, the assay system was validated by comparing the relative susceptibilities of *A. castellanii* and *A. polyphaga* to chlorhexidine over a 4-day period and comparing the results obtained with those obtained by the widely used manual counting method. The results obtained by both methods not only were in agreement with each other but also demonstrated that *A. polyphaga* is more resistant to chlorhexidine than *A. castellanii*, irrespective of the assay used. Chlorhexidine is known to be a membrane-active agent and to target acidic phospholipids, although other mechanisms, such as inhibition of ATP synthesis, have been proposed to occur in the presence of high chlorhexidine concentrations (15).

To further evaluate the utility of the assay that was developed, the susceptibilities of *A. castellanii* and *A. polyphaga* to APC and AGPC, phospholipid analogues, were assessed. A previous study (22) examined the effects of a number of APCs on *Acanthamoeba* species by a manual counting assay, but until now, AGPCs had yet to be examined. In agreement with the previous study (22), using the alamarBlue assay we found that hexadecyl-PC (16-carbon chain length) was completely effective (IC$_{100}$) at similar concentrations against both *A. castellanii* and *A. polyphaga*. Similarly, we found octadecyl-PC (18-carbon chain length) to be less effective, an effect that was exemplified with *A. polyphaga*. Longer chain lengths with various degrees of saturation were tested and, with the exception of (*Z,Z*)-6,12-eicosadienylphosphocholine, were found to be largely ineffective at a concentration of 80 μM (22). We therefore examined the effect of edelfosine, an AGPC with an 18-carbon chain length. Our results indicate that this compound is poorly effective. Previous studies (10) have demonstrated that edelfosine is effective against *Leishmania donovani* and a number of other trypanosomatids at low-micromolar levels (<5 μM for *L. donovani*). This would indicate that *Acanthamoeba* species are susceptible to a more limited range of the available phospholipid analogues than the trypanosomatids. Furthermore, the relative differences in the susceptibilities of *A. castellanii* and *A. polyphaga* to chlorhexidine, octadecyl-PC, hexadecyl-PC, and edelfosine observed in this study suggest differences in the phospholipid compositions of their membranes.

A challenge in the field of *Acanthamoeba* research will be to

develop antimicrobial agents better able to destroy the cyst stages. Our unpublished results suggest that the cyst stages are not highly metabolically active and that the alamarBlue assay is unlikely to be useful in enumerating these stages directly. However, an adaptation of the assay could be envisaged for this purpose. For this, cyst stages would be exposed to potential inhibitors for a set period of time, after which the growth medium would be replaced by fresh medium that lacked inhibitors. The number of viable cysts able to transform into trophozoites could then be assessed by their ability to reduce alamarBlue.

In conclusion, we have developed a colorimetric, microtiter plate assay based on the commercially available alamarBlue reagent to test the activities of antimicrobial compounds against *Acanthamoeba*. This method offers a number of advantages over many of the systems used at present. It is rapid, the results can be read in a nonsubjective manner, the assay measures only live cells, and minimal hazards are associated with the reagents used. The low seeding requirements of a 96-h study means that a single 75-cm^2 flask containing 10^7 cells provides sufficient numbers of *Acanthamoeba* cells to assay more than 3,000 samples in triplicate. As it is based on a 96-well microtiter plate, it could easily be automated and is amenable to scaling for high throughput.

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