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Research Article

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Standardizing the assessment of amoebicidal efficacy of contact lens solutions against *Acanthamoeba* species

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ABSTRACT

To date no standardized methods are used in order to assess the amoebicidal efficacy of commercial contact lens solutions for both trophozoites and cysts of Acanthamoeba species. *Here we present two methods that are suitable for this purpose: The Spearman-Karber log reduction method and XTT colorimetric assay. Acanthamoeba castellanii (ATCC 50370) and A. polyphaga (ATCC 30461) trophozoites were cultured in peptone-yeast extract-glucose medium. Cysts were developed in Neff's encystement medium for 1 week. Spearman-Karber and XTT colorimetric assay were used to evaluate trophozoite and cystocidal efficacy of Multi-Purpose Contact Lens Solutions (MPS).With trophozoites, the Spearman-Karber method gave a log reduction estimate of morphological kill between log 0.83 and log 3.61 of the various contact lens solutions, enabling the differentiation between efficacious and less efficacious solutions. With cysts the maximum log reduction of 2.17 was achieved for all 3 MPS solutions at 8 hours. The XTT colorimetric assay showed reduction in trophozoite metabolic rates between 50 and 100% as provided by an optical density signal. All lens fluid solution with a reduction rate >90% showed residual growth of Acanthamoeba after one week of incubation on nutrient agar covered with Enterobacter aerogenes. Both methods give reproducible estimates of amoebicidal efficacy of contact lens solutions, however, XTT colorimetric assay should be followed by an assay for residual growth to test for viable cysts.*

Keywords: *Acanthamoeba* Species; Lens Fluid Solutions; Efficacy; Colorimetric Assay; Log Reduction Method

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INTRODUCTION

There has been an increase in the incidence of *Acanthamoeba* Keratitis (AK) associated with contact lens wear over the last decades [1,2]. AK is an infection of the cornea due to *Acanthamoeba* species and is associated with the use of soft Contact Lenses (CL). Personal hygiene is an important factor when using contact lenses and cleaning by rubbing and rinsing the lenses [3], and disinfecting them with all-in-one CL solutions, hydrogen peroxide or povidone iodine CL solutions is strongly advised [4,5]. Over the past decades the distribution of CLs and CL solutions has been made available also to general drugstores and internet. Therefore, the supervision of CL use has been shifting from specialized opticians to unsupervised use [6,7]. Possibly the increased availability and use of unsupervised CL is an important factor in the increase in AK patients seen by cornea specialists [2]. Risk factors like rinsing lenses and CL cases in tap water, swimming and / or while wearing CL and using multipurpose solution for cleaning the lenses have also been forwarded [8]. Further, as was reviewed by Bradley et al. it has been repeatedly shown that CL solutions are not efficient enough in the killing of *Acanthamoeba* species, especially not the cyst life stage [9]. *Acanthamoeba* are free-living amoeba that can be found everywhere, such as in tap water, surface waters, the air, soil and in vegetables. It consists of 23 genotypes (T1-T23), which are subdivided based on the nucleotide sequence of the 18S *rRNA* gene [10]. The most common genotype causing keratitis is T4. *A. castellanii* and *A. polyphaga* belong to this genotype [11]. The life cycle of *Acanthamoeba* consists of two stages: trophozoite and cyst. Trophozoites are the metabolically active stage in which the amoeba replicate and may infect individuals. Cysts are the metabolically inactive stage in which the trophozoite transforms to protect itself from adverse environmental conditions. The cyst stage has a double cell wall consisting of an ectocyst and endocyst, which is known to be much more resistant to disinfectants [12].

Before market release, CL solutions are tested according to an International standard protocol that describes European quality regulations, ISO 14729 [13]. However, this protocol only tests for bacteria and fungi killing. The protocol argues that testing for *Acanthamoeba* species is not essential as AK keratitis is very rare and a lens care system that eliminates contamination with bacteria will also reduce the incidence of *Acanthamoeba* contamination to a large extent. It has been argued that cysts, being the dormant stage, are not infectious. Therefore, inducing encystment by lens fluid solutions could be an effective preventive measure against AK. For assessing CL solution efficacy inducing *Acanthamoeba* trophozoite encystment, the protocol of trophozoite encystement was developed and described in the European quality regulations ISO 19045 [14]. This protocol does not describe a method to assess the efficacy of the disinfecting components in contact lens solutions against trophozoites and cysts of *Acanthamoeba* species, as it only aims to measure the encystment rate. However, after encystment *Acanthamoeba* cysts can still revert to trophozoites. Therefore also cysts should be considered an extra risk for AK development and contact lens solutions should be effective against both cyst and trophozoites, as has also been argued by others [15].

The present study describes two *in-vitro* assays suitable for assessing the amoebicidal efficacy of CL solutions. The Spearman-Karber method [16,17] is suitable for testing amoebicidal efficacy against both trophozoites and cysts of *Acanthamoeba* species. The XTT-colorimetric assay [18,19] followed by a residual growth assay, will test the amoebicidal efficacy against trophozoites and viable cysts.

MATERIALS AND METHODS

Trophozoite and cyst culture

We selected two subtypes of *Acanthamoeba* species for this study as different subtypes may have various biological characteristics. *A. castellanii* (ATCC 50370) and *A. polyphaga* (ATCC 30461) trophozoites were grown in peptone-yeast extract-glucose (PYG) medium comprising: 0.98 g $MgSO_4 \cdot 7\ H_2O\ L^{-1}$; 1 g $C_6H_5Na_3O_7 \cdot 2\ H_2O\ L^{-1}$; 0.02 g $Fe(NH_4)_2(SO_4)_2 \cdot 6\ H_2O\ L^{-1}$; 0.34 g $KH_2PO_4\ L^{-1}$; 0.355 g $Na_2HPO_4 \cdot 7\ H_2O\ L^{-1}$; 20 g proteose peptone L^{-1}; 2 g yeast

extractL^{-1}; 18 g *D*(+)-glucose-monohydrate L^{-1} and 0.059 g $CaCl_2 \cdot 2\ H_2O$ l^{-1} of deionized water. The pH was adjusted to 6.5 ± 0.2. Eight mL PYG medium was transferred to T25 tissue culture flasks, an aliquot of trophozoites was added and the flask was incubated at 28˚C.

A. castellanii (ATCC 50370) and *A. polyphaga* (ATCC 30461) cysts were produced using Neff's encystement (NEM) medium comprising: 7.46 g KCl L^{-1}; 0.96 g $MgSO_4$ L^{-1}; 0.04 g 2-amino-2-methyl-1,3-propanediol L^{-1} of deionized water. The pH was adjusted to 7.4-7.8 and after 5 hours to 9.0 with 1 M NaOH. To produce cysts, the trophozoites were harvested at a concentration of 1 to 2 × 10^7 trophozoites per mL through centrifugation at 780 rcf for 5 minutes. The pellet was washed three times with ¼ Ringer's solution (Thermo Fischer Scientific, Hampshire, IK) and afterwards inoculated into 8.0 mL of Neff encystment medium for 7 days at 28˚C. Amoebic concentrations were estimated by counting trophozoite and cysts using Bürker Türk cell counting chambers (Faust Laborbedarf AG, Schaffhaussen, Germany).

Test solutions

In order to illustrate these two methods, a selection of three multipurpose CL solutions (MPS) was made from a more extensive study (2). The main biocidal components in each MPS were: MPS 1 (0.0001% PHMB), MPS 2 (0.00016% Alexidine, 0.0003% Polyquaternium) and MPS 3 (0.001% Polyhexanide). The recommended disinfection times (hereafter RDT) for MPS 1 and MPS 3 were 4 hours and that of MPS 2 was 6 hours. All the CL solutions bottles were previously unopened and used within two weeks after opening.

Spearman-Karber log reduction method

Spearman-Karber computations were used to determine the level of *Acanthamoeba* kill in relation to exposure to the different contact lens solutions. ¼ Ringer's solution was used as reference (R = amoeba not exposed to CL solution) [16].

The procedures described hereafter were performed for both *A. castellanii* and *A. polyphaga*. 4.95 mL of each multi-purpose contact lens solution (MPS) or ¼ Ringer's solution was transferred to a large Greiner tube. Fifty µl of 5 × 10^6 cells mL^{-1} trophozoites or cysts were added to each Greiner tube in order to obtain a final concentration of 5 × 10^4 amoebic cells mL^{-1}. Tubes with MPS CL solution and amoeba were incubated 0, 4, 6 and 8 hours at 28˚C before the tubes were mixed thoroughly by vortex. Half a mL of each Greiner tube was transferred to 4.5 mL Dey-Engley Neutralizing Broth (DENB) in order to neutralize the exposure to MPS, making the 0-hour tubes a Positive Control (PC).

Following, tenfold serial dilutions of each DENB tube were made with PYG medium (theoretical concentrations of 5 × 10^3, 5 × 10^2, 5 × 10^1 and 5 × 10^0 cells mL^{-1}). Two hundred µl per well from the serial dilutions was transferred to a 96 wells plate in triplicate. Hundred-and-twenty µl per well of the positive control was also transferred together with 80 µl per well PYG medium. The 96 wells plates were incubated at 28˚C, with the incubation period depending on the stage of the *Acanthamoeba*: 1 week for trophozoites and 2 weeks for cysts. After the incubation period, each well was assessed for growth by visual examination using a phase contrast microscope. The number of positive wells (wells with at least 2 amoeba growing) were counted and the log reduction of each test solution was calculated using the Spearman-Karber equation [16].

XTT colorimetric assay

The procedure described hereafter was performed for both *A. castellanii* and *A. polyphaga* and repeated in order to have a beginning stock for each MPS. Trophozoites were seeded in a 96 wells plate in triplicate at 20,000 amoeba per well in 200 µl per well ¼ Ringer's solution. A serial dilution of 20,000, 10,000, 5,000, 2,500 and 1,250 amoeba

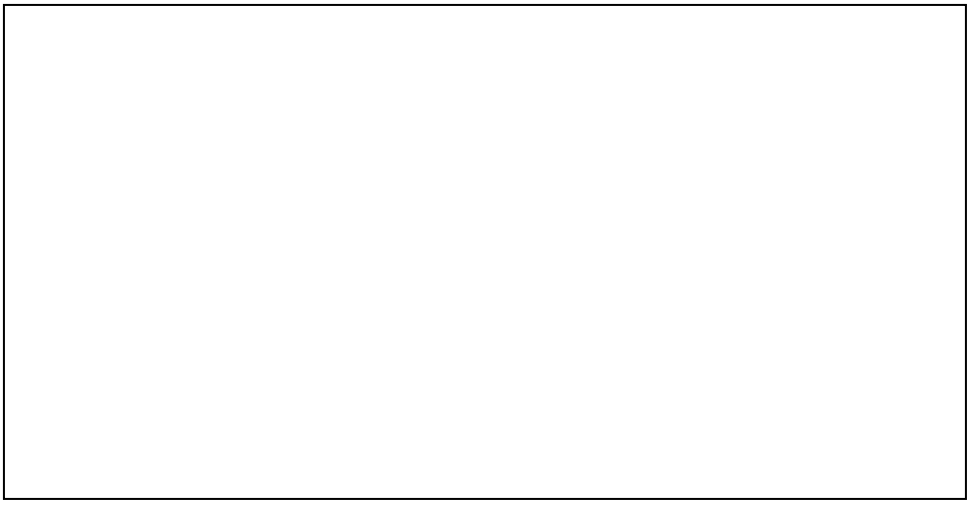

Figure 1: Mean optical density (OD) of amoeba in MPS related to mean number of amoeba in a serial dilution. Say mean OD of triplet MPS after 8 hours incubation with MPS is 0.036 corresponding to ~5000 amoeba/ml according to the serial dilution. The % reduction is then 100–((5000/20000) × 100) =75%. Details of XTT colorimetric assay. Note: : Mean OD of triplet serial dilution; : **Mean OD of triplet MPS.**

Statistical methods

The data was analysed using GraphPad Prism (Version 6.0; GraphPad software, Inc, San Diego). Differences were considered statistically significant at $P \leq 0.05$. The data was analysed through one way ANOVA together with a Dunnett's test. Amoebicidal efficacy was expressed as log reduction of growth when using the Spearman-Karber-method and percentage of reduction in optical density, when considering the XTT-colorimetric method.

RESULTS AND DISCUSSION

Spearman-Karber method

The efficacy against trophozoites (Figures 2a and 2b): MPS 1 and MPS 3 were relatively ineffective against *A. castellanii* trophozoites. At the Manufacturer's Recommended Disinfection Time (MRDT), the log reduction of MPS 1 and 3 was not significant different from that of PC. After 8 hours, a maximum log reduction of 0.83 and 1.94 was achieved for MPS 1 and 3, respectively. MPS 2 did show a significant amoebicidal effect at MRDT when compared to PC, with a log reduction of 3.50. After 8 hours, this improved further to a log reduction of 3.61. For *A. polyphaga* trophozoites, each MPS were significant more amoebicidal than PC at MRDT, however none of the MPS exceeded a log reduction of 3.0. After 8 hours, the log reduction ranged between 2.06-2.83 for all three MPS. Note: a log reduction of 4.50 is the highest achievable as this corresponds to 100 % efficacy (=no growth).

The efficacy against cysts (Figures 2c and 2d): The log reduction after exposing *Acanthamoeba* cyst to the three MPS or ¼ Ringer's solution, showed less variability. Considering *A. castellanii* cysts, the log reduction for any of the MPS did not surpass 2.17. MPS 1 showed no significant difference with PC at MRDT, and at 8 hours a maximum log reduction of 1.50 was achieved. Both MPS 2 and MPS 3 showed a significant better log reduction at MRDT when compared to PC (1.39 and 1.61 versus <1.0, respectively). After 8 hours, the maximum log reduction ranged between 1.83 and 2.06. Considering *A. polyphaga* cysts, the log reduction of MPS 1 and 3 at MRDT did not differ significantly with that of PC (1.50 and 1.94 versus 1.28 respectively). A maximum log reduction ranging between 1.61 and 2.17 was achieved for MPS 1 and 3 at 8 hours. Considering MPS 2, the log reduction was

Spearman-Karber log reduction and XTT colorimetric assay were performed to assess the amoebicidal efficacy of three contact lens solutions with varying amoebicidal efficacy against two different *Acanthamoeba* species. Figure 2 shows the results of the Spearman-Karber method and Figure 3 shows the results of XTT colorimetric assay. The Spearman-Karber log reduction method provides a visual observation of the amoebicidal efficacy of each lens fluid. The XTT colorimetric assay provides information about reduction in metabolic activity which does in fact not reflect complete kill of trophozoites and cysts as cysts are metabolic inactive. Part of the reduction in metabolic activity can be explained by parasite encystation. We showed residual growth of amoeba even though reduction rates were >90%.

As the incidence of *Acanthamoeba* keratitis (AK) has increased over the past decades, the question came up once more, if CL solutions show sufficient amoebicidal effectivity. Quality control procedures, based on the ISO 14729 [13], describe how to examine lens fluid bactericidal and fungicidal efficacy. However, this protocol deliberately does not contain criteria regarding the effectiveness against *Acanthamoeba* species as there is no standard method of testing the efficacy of lens solutions against *Acanthamoeba* species and authorities have argued that if solutions are bactericidal, *Acanthamoeba* cannot grow as there are no bacteria to feed on. Still, in clinical laboratory practice we are increasingly confronted with *Acanthamoeba* contaminated contact lenses and storage cages over the past years, possibly due to decreasing professional supervised CL wear [6,7].

We developed a standardized protocol describing two *in-vitro* susceptibility assays which assesses the amoebicidal efficacy of contact lens solutions for both trophozoites and cysts. It was difficult to produce homogeneous results. Therefore, procedures were always performed in triplet taking continuous care of good mixing as amoeba tend to adhere to test tube surfaces causing variation in serial dilutions made [20]. Furthermore observations were subject to inter-observer variation. Therefore, when assessing the presence or absence of amoebic growth, we assumed that the presence of at least two trophozoites or cysts in the well, defined growth. The literature does not designate the criteria for a positive or negative assessment of the wells [4,21].

We observed a significant difference in lens fluid amoebicidal activity between the two subtypes tested in our experiment, *A. castellanii* and *A. polyphaga*. The variation between subspecies has also been described by others [4, 17]. It seems that *A. polyphaga* shows less variation to the disinfecting effect of various CL solutions. This observation was made by Spearman-Karber method as well as by the colorimetric method. However, there may be a methodological bias as we had to modify the colorimetric protocol when testing *A. polyphaga*. The XTT incubation time in this assay was increased in experiments with *A. polyphaga* due to the slower metabolic rate of this subspecies. The reduction of XTT is based on the citric acid cycle. When a strain is less metabolically active, NADH is produced at a slower rate. Consequently, XTT will be converted less quickly. It therefore appears that diverse *Acanthamoeba* strains need different incubation times in the XTT colorimetric assay depending on their metabolic rate. No methodological adjustments were made in the Spearman-Karber method when testing different subtypes. This observation indicates that different *Acanthamoeba* species vary in their sensitivity to contact lens solutions, which, in itself, is an interesting observation as we do not know which subspecies is causing AK in the Netherlands.

Spearman-Karber method of susceptibility testing is a classical bioassay also described in virology and used in order to estimate EC_{50} and EC_{90} against virucidal and amoebicidal components elsewhere [16]. However, this method is laborious and subject to inter-observer variability. Therefore, we introduced the XTT colorimetric assay, a method that is more suitable for high-through-put testing. Although a trend to identical results may be present, the methods yielded different results. A reduction in OD of 90% (colorimetric assay) would theoretically correspond to a log reduction of 1.0 (Spearman-Karber method), an OD reduction of 99% corresponds to log reduction of 2.0, and an OD reduction of 99,9% corresponds to log reduction of 3.0. We observed higher efficacy using the Spearman-Karber method compared to the colorimetric assay. However, trophozoites and cysts were tested separately in the Spearman-Karber method, allowing higher efficacy measures in the experiments with trophozoites which are known