

# Viability test exclusively is not adequate to evaluate the T4 *Acanthamoeba* keratitis' treatment

## Abstract

*Acanthamoeba* keratitis is a serious corneal infection and can lead to blindness. Cysts and recurrence of infection represent a challenge in the treatment of keratitis due to its high resistance to adverse conditions and to most medications. This intriguing property can lead to late diagnosis and can explain absence of cyst removal during and after therapy regimen. So the evaluation of *Acanthamoeba* cysts viability after exposure to drugs used in *Acanthamoeba* keratitis treatment is so important.

**Material and methods:** Cysts (10<sup>5</sup>/mL) of two clinical and an environmental T4 isolates were separately incubated with polyhexamethylene biguanide (0.02%), chlorhexidine digluconate (0.02%) or propamidine isethionate (0.1%) at 37 °C for a period of 1h or 24h. Cysts were quantified, stained with trypan blue and inoculated in agar plates covered with inactivated *Escherichia coli*.

**Results:** None of the treatment regimens induced complete cysts elimination. The environmental isolate had no reduction on cysts quantification compared to clinical isolates. The trypan blue exclusion method demonstrated non-viable cysts in the treatment with chlorhexidine digluconate and propamidine isethionate. In the treatment with polyhexamethylene biguanide, viable and non-viable cysts were observed after 24h of incubation. The cultures were positive for all treatment protocols except for clinical isolate ATCC 30461 with chlorhexidine digluconate regimen after 24 h.

**Conclusions:** The presence of cysts observed in cultures reiterates cysts viability even after treatment demonstrating the need to follow up this form of development during treatment. The trypan blue staining is not adequate to evaluate the *Acanthamoeba* cysts viability due to its recovery in culture. With these results, we conclude that the viability test exclusively is not adequate to evaluate the *Acanthamoeba* keratitis treatment and that its always necessary to perform culture technique.

**Keywords:** *Acanthamoeba*, viability, culture, polyhexamethylene biguanide, chlorhexidine digluconate, propamidine isethionate, trypan blue

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## Introduction

*Acanthamoeba* keratitis is a serious corneal infection and in some cases can lead to blindness.<sup>1</sup> The T4 genotype is often associated with human infections by *Acanthamoeba*.<sup>2,3</sup> The incidence of keratitis has increased considerably worldwide, especially those associated to contact lenses use.<sup>4,5</sup> Cysts and recurrence of infection represent a challenge in the treatment of keratitis due to its high resistance to adverse conditions and to most medications.<sup>6,7</sup> This intriguing property can lead to late diagnosis and can explain absence of cyst removal during and after therapy regimen.<sup>8</sup> In early stage, due to symptomatology and incorrect diagnosis the disease can be inappropriately mistaken for keratitis by other microorganisms, such as fungi and bacteria, leading to treatment with antifungals or antibiotics that will not be effective. Therefore, delaying proper treatment for this infection can increase severity and risk of blindness.<sup>9</sup>

Although most available treatments are effective against trophozoites, cysts represent a problem due to the recurrence of infection even after correct therapeutic approach.<sup>10</sup> *Acanthamoeba* has a worldwide distribution.<sup>11</sup> Cysts are resistant to drugs due to their double layer membrane that confers protection against most drugs. The use of cysts in *in vitro* studies allows evaluation of different amebicidal agents effectiveness and favors the indication of potential therapies for *Acanthamoeba* keratitis.<sup>12</sup> Polyhexamethylene biguanide (PHMB)

or chlorhexidine digluconate (CLX) are the drugs currently used in keratitis treatment, associated or not with propamidine isethionate (PROP) or hexamidine.<sup>13,14</sup> These drugs are used as eye drops (topical use). At the beginning of the treatment regimen administration is done every 1 hour during 48 hours. After this interval, the treatment extends for another 72 hours with one administration daily. As the intensity of use is toxic to the corneal epithelium, administration is reduced after the first few days and individually adapted according to the patient's need.<sup>1,15</sup> Therefore, the aim of this study was to evaluate *Acanthamoeba* cysts viability from clinical and environmental isolates in the same treatment regimen used for keratitis and under monotherapy.

## Material and methods

### Isolates

The isolates used in this study were: standard strain ATCC 30461 (*Acanthamoeba polyphaga*),<sup>16</sup> BsB6 (from keratitis)<sup>17</sup> and IP1S1 (from swimming pools).<sup>18</sup> All isolates belong to T4 genotype.<sup>16-18</sup>

### *Acanthamoeba* cysts

In order to obtain cysts, cultures were performed on plates containing non-nutrient agar with *Escherichia coli* previously inactivated by heat (60°C for 4 h). The plates were incubated at

28°C and 37°C and observed for 2 months.<sup>16,19</sup> All plates containing cysts were washed with sterile saline (0.9%) to remove cysts from the surface.<sup>20</sup> The content was removed and stored at 4°C for hemocytometer quantification and drug exposure.

### Drugs exposure

The cysts concentration was adjusted to 10<sup>5</sup> parasites/ mL<sup>21</sup> and exposed to 1mL of polyhexamethylene biguanide (0.02%), chlorhexidine digluconate (0.02%) or propamidine isethionate (0.1%) in separate tubes<sup>22</sup> in 1:1 ratio. After a period of either 1h or 24h incubation at 37°C, cysts viability was analyzed. For the control group, incubation with water for injection was used. After drugs exposure, hemocytometer quantification of evolutionary forms was performed. All experiments were carried out in triplicate.

### Cysts viability

The viability assays were performed using the trypan blue (0.4%) exclusion method at 1:1 ratio in *Acanthamoeba* cysts after drugs exposure. Stained cysts (non-viable) and non-stained (viable) cysts were quantified in hemocytometer.<sup>23</sup>

### Cysts recovery

After drugs exposure, a 200 µl aliquot of each sample was added to the plates containing non-nutrient agar with *E. coli*, incubated at 37 °C and monitored for 7, 14 and 21 days in order to perform culture recovery.

### Statistical analyzes

The t-test was used for comparison between the initial inoculum (10<sup>5</sup> cysts/ mL) and the final quantification after drugs exposure and the ANOVA test was used for comparison between the final quantifications after different treatments regimen. The statistical analysis was performed with Sigma Stat 3.0 software and the difference was considered statistically significant when  $p < 0.05$ .

## Results

After 24 hours of exposure, there was reduction in cysts quantification in the ATCC 30461 isolate both in the groups treated with PHMB, CLX and in the control group ( $p < 0.05$ ) compared with the initial inoculum (Figure 1B). In the BsB6 isolate there was reduction in cysts quantification in all treatments regimen, including the control group ( $p < 0.05$ ) (Figure 1F). Regarding the 1h treatment period, cysts quantification reduction was observed in ATCC when exposed to PROP ( $p < 0.05$ ) (Figure 1A). In BsB6 and IP1S1, this reduction was not observed in all treatment approaches (Figure 1C and 1E).

In the treated groups belonging to IP1S1 isolate, when compared with the initial inoculum (10<sup>5</sup> cysts/mL), there was no reduction of cysts quantity at the two proposed time intervals (Figure 1C, 1D). In this same isolate, when comparing the different treatment approaches, it was observed that cysts quantification in 1 hour of exposure was higher in the control group when compared with PHMB and PROP ( $p < 0.05$ ). With CHX, the cysts quantification was the same as the control group ( $p > 0.05$ ) (ANOVA test) (Figure 1C).

### Viability analysis

In the analyzed samples, stained (non-viable) cysts and non-stained (viable) cysts were observed. In the 1h exposure period stained

cysts (non-viable) were observed in all isolates and in all treatment regimens. Regarding the 24h treatment period, heterogeneity (presence of both stained and non-stained cysts) was observed only in the groups treated with PHMB for 24h, which presented viable and non-viable cysts in all isolates. Samples incubated with water for injection showed uncolored cysts, which indicated the control groups cysts were viable (Figure 2A). In the remaining treated groups only stained (non-viable) cysts were observed (Figure 2B).

### Recovery in culture

After drugs exposure, isolates presented positive cultures, with exception of ATCC 30461 exposed for 24 hours to CLX. As expected, all control groups cultures were positive.

## Discussion

This study analyzed the resistance of *Acanthamoeba* T4 genotype to drugs used in keratitis treatment. Only the IP1S1 (swimming pool) isolate showed no reduction in cysts concentration after the treatment regimen. Sunada et al.,<sup>24</sup> in a study with clinical isolates exposed to PHBM, CHX and PROP, obtained similar results, in which isolates were more susceptible after 24h exposure. These authors also pointed out the low susceptibility of clinical isolates to drugs. In another study, *Acanthamoeba castellanii* cysts (ATCC 30868) were also susceptible when exposed during 24h to PHMB (0.02%) and PROP (0.1%).<sup>25</sup>

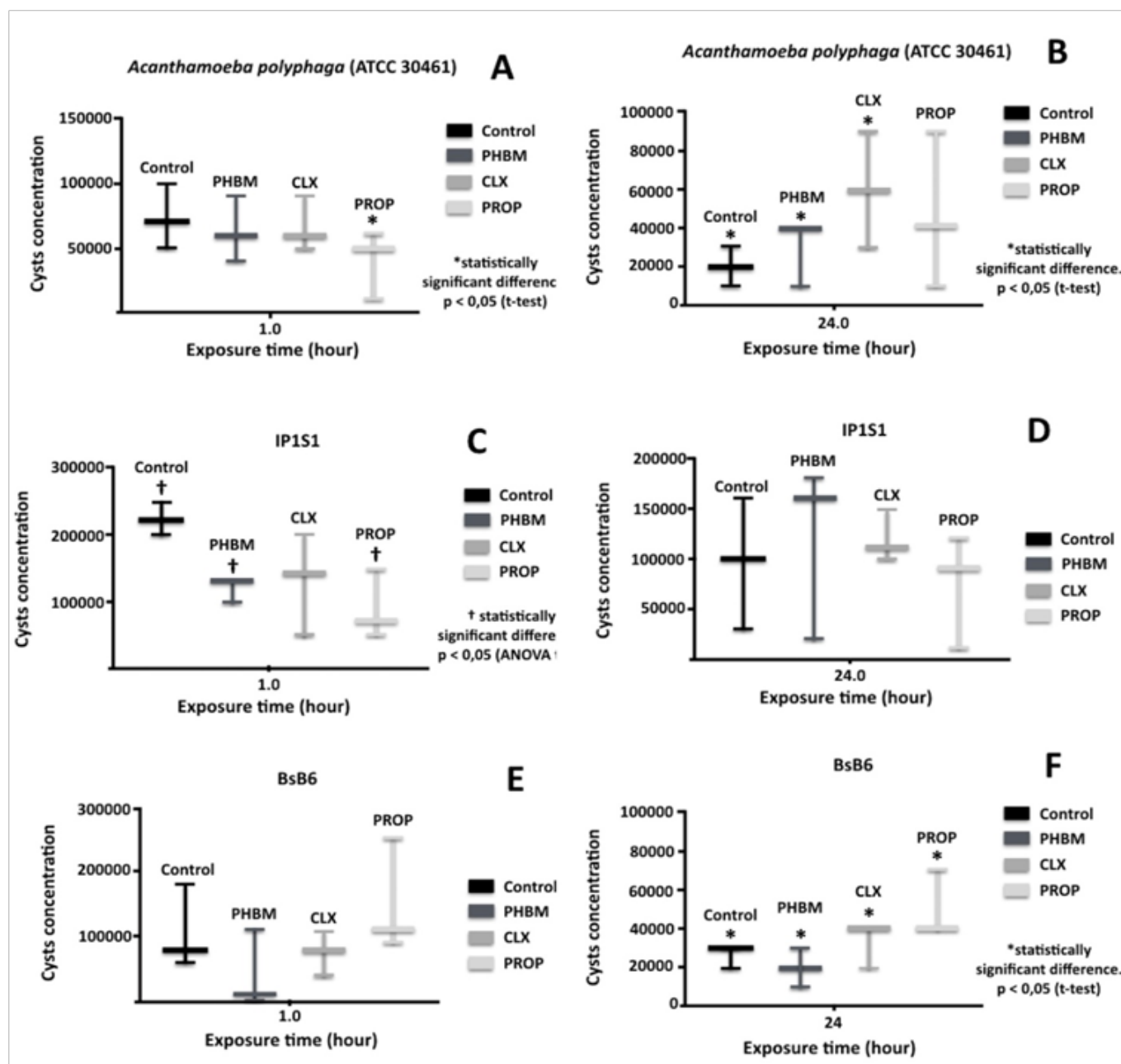
Only the IP1S1 (swimming pool) isolate showed no reduction in cysts quantification after the treatment regimens (1h and 24h). The increase in cysts quantification in control group (1h) of IP1S1 isolate could also be related to the resistance of this isolate previously described by our team as invasive<sup>26</sup> demonstrating the ability of its trophozoites to multiply from the initial inoculum, which would make it difficult to reduce the cysts concentration by drugs action. One of the risk factors for keratitis is the practice of swimming wearing contact lenses.<sup>27</sup> It was seen that *Acanthamoeba* sp. cysts from tap water were resistant when exposed to chlorination<sup>28</sup> and contact lens cleaning solutions.<sup>29</sup> Therefore, it is important to emphasize the resistance of environmental isolates to disinfection methods and reinforce the need for correct contact lenses handling to reduce the possibility of contamination and the risk of *Acanthamoeba* infection.

In BsB6, regarding the 1h period, there was no significant difference in the reduction of cysts quantification after exposure to treatment regimens. This clinical isolate<sup>17</sup> did not present a reduction in the concentration of its resistant form despite treatment used for keratitis. It was observed that PROP was able to reduce cysts concentration in ATCC after 1h exposure. The T4 genotype is commonly associated with keratitis, however, there are attempts to differentiate isolates from the same genotype and it is suggested there are subtypes with probable genetic variation among T4 complex clones.<sup>30</sup> It is important to consider this fact while studying the behavior of different *Acanthamoeba* isolates belonging to this genotype.

The use of these drugs without association allowed cysts concentration reduction after 24h. When PHMB or CHX were associated with diamidine, they exhibited a presumed anti-amebial additive effect.<sup>13,31</sup> However, our results showed monotherapy is more effective in reducing parasite's concentration in culture and that exposure time is crucial for successful treatment. Therefore a triage to aid the choice of the correct drug effective against each kind of keratitis infection is essential, such as an antibiogram and the correct administration during optimal time intervals will contribute

to eradicate the infection. In the groups treated with CLX and PROP without association, using the 0.02% concentration during the two time intervals, the observation of non-viable cysts was possible. In a study that also used trypan blue exclusion method a low percentage of viable cysts was observed when PHMB and CLX were associated

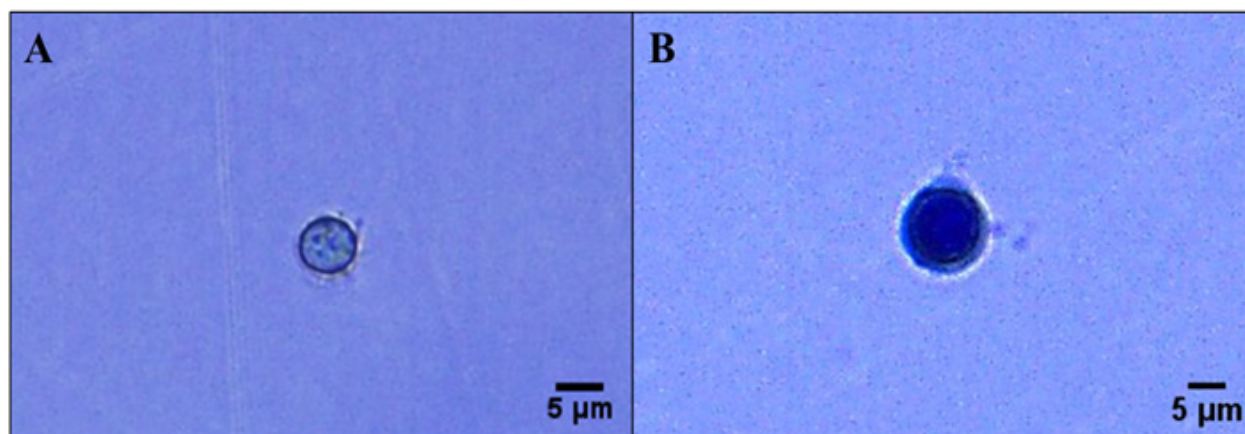
with 0.04% concentration in two drops of each drug after a period of 48 hours exposure.<sup>32</sup> In this study, the exposure conditions could have enabled sufficient interactions of the compounds with the cysts to allow absorption of the trypan blue.



**Figure 1** *Acanthamoeba* cysts after drugs exposure during 1h or 24h. Inoculum: 105 cysts/mL. A and B) *Acanthamoeba polyphaga* (ATCC 30461); C and D) PIS1 isolate (swimming pools); E and F) BsB6 (clinical sample). Graphs: minimum, maximum and median of the triplicate. CLX, chlorhexidine digluconate; PHBM, polyhexamethylene biguanide; PROP, Propamidine isethionate

\*  $p < 0.05$ , Comparison between the inoculum 105 cysts/mL and the final quantification cysts after drugs exposure (t-test).

†  $p < 0.05$ , Comparison between the final quantification of the different treatments (ANOVA test).



**Figure 2** *Acanthamoeba* sp cysts of IPISI isolate under trypan blue staining. A, control group showing non-stained (viable) cyst; B, after 1 hour exposure to chlorhexidine digluconate showing stained cyst (non-viable). Optical Microscope. 400x.

CLX is a cationic compound that acts on the cytoplasmic membrane<sup>33,34</sup> and PROP belongs to the group of diamidines that act as a cationic surfactant promoting membrane rupture, amino acids leakage and oxygen uptake inhibition.<sup>35</sup> The drugs interaction mechanisms in *Acanthamoeba* cysts have not yet been fully elucidated and the existing information is based on studies with other microorganisms such as fungi, bacteria or other protozoa.<sup>36</sup> It is possible that drugs interaction in cysts wall allowed vital dye entry inside the cysts treated with CLX and PROP and PHMB during 1h period, in which the cysts were stained.

This study showed positive cultures after drugs exposure. In contrast, it has been previously reported by Wysenbeek et al.,<sup>37</sup> that *Acanthamoeba* cysts from keratitis isolates, exposed during 24 hours to CLX at 0.1% concentration, showed negative results in culture. However, it was reported high doses of CLX (4%) was associated to high cytotoxicity, which is harmful to corneal and conjunctival cells, and low concentrations (0.005%) led to fewer corneal side effects. The concentrations used for these first-line drugs are based on clinical cases and laboratory analyzes, however, these compounds still have corneal toxicity and patients should be followed up.<sup>22,38</sup> It has been recommended *Acanthamoeba* culture should be monitored during at least for three weeks, as performed in this study, since some strains and isolates take a longer period to exhibit growth.<sup>15</sup>

The cultures positivity in which cysts concentration reduction was observed after the quantification procedures indicate that *in vitro* time exposure and drugs concentrations used were not sufficient to eliminate all cysts from the initial inoculum. This data shows the utmost importance of correct drug triage before initiating keratitis treatment as well as the choice of correct treatment regimen.

Cysts resist adverse conditions such as freezing, pH changes, differences in osmolarity, and chemical compounds.<sup>25</sup> They may also survive in culture for more than 20 years in extreme dry conditions, such as desiccation.<sup>39</sup> It is noteworthy that after 24h exposure, the clinical isolate BsB6 presented reduction in cysts quantification and positive cultures in all treatment regimens. The same was observed in ATCC 30461 isolate after PHMB and CLX treatment regimens. In about 10% of amoebic keratitis cases recurrence is inevitable.<sup>36</sup> The presence of cysts observed in cultures reiterates cysts viability even after treatment.

## Conclusion

Our study showed that quantification techniques and culture recovery were capable to evaluate *in vitro* the method for keratitis treatment. The presence of cysts observed in cultures reiterates cysts viability even after treatment demonstrating the demonstrating the need to follow up this form of development during treatment. The trypan blue staining is not adequate to evaluate the *Acanthamoeba* cysts viability due to its recovery in culture. With these results, we conclude that the viability test exclusively is not adequate to evaluate the *Acanthamoeba* keratitis treatment and that it's always necessary to perform culture technique.

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## Conflicts of interest

Authors declare that there are no conflicts of interest.

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