OPHTHALMOLOGY

Anti-*Acanthamoeba* Activity of Oxysept® Hydrogen Peroxide System against Cysts of *Acanthamoeba* from Environmental Isolates

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ABSTRACT

Introduction: **Keratitis caused by the free-living *Acanthamoeba* is one of the most difficult ocular infections to manage successfully due to the resistance of the organism's cyst stage to most antimicrobial agents. As this condition occurs mainly amongst the contact lens wearers, a contact lens disinfecting solution which is not effective against the *Acanthamoeba* cyst, may not protect the wearers against its infection.**

Objective: **This study investigates the cysticidal efficacy of Oxysept® hydrogen peroxide system against two environmental isolates of *Acanthamoeba* from Malaysia.**

Materials and Methods: **Soaking of the *Acanthamoeba* cyst isolates (TTT1 & TKT19) suspension to Oxysept® hydrogen peroxide system was performed to determine the anti-*Acanthamoeba* activity of the contact lens disinfecting solution. The soaking times were based on the manufacturer's recommended time (6 hours), 4 hours and over-night soaking for 8 hours. The cysticidal efficacy of the contact lens disinfecting solution was determined by detecting the presence of *Acanthamoeba* trophozoites on the non-nutrient agar (NNA) plates lawned with heat killed Escherichia coli following disinfection. The emergence of trophozoites from the cysts that had been soaked with Oxysept® hydrogen peroxide system indicates that the system is not effective against *Acanthamoeba*.**

Result: **Oxysept® hydrogen peroxide system against *Acanthamoeba* cysts gives varying results. The manufacturer's recommendation of mixing of the disinfectant simultaneously with neutralizing tablets, exhibited no cysticidal effect. However, when the cysts were soaked in hydrogen peroxide solution only without the neutralizing tablet, it exhibited cysticidal effect against *Acanthamoeba* cysts at all soaking periods.**

Conclusion: **This study concludes that Oxysept® hydrogen peroxide system shows anti-*Acanthamoeba* activity if it is not used simultaneously with the neutralizing tablet. Therefore the disinfection process should be allowed to take place for some period of time before it is neutralized to achieve the cysticidal effect and thus help prevent *Acanthamoeba* keratitis.**

KEY WORDS

***Acanthamoeba*, contact lens disinfecting solution, hydrogen peroxide, Malaysia**

INTRODUCTION

Microbial keratitis is a serious vision-threatening infection. Clinical features include redness, pain, tearing, blur vision and inflammation but symptoms vary depending on the causative agent. *Acanthamoeba* species are the major cause of parasitical keratitis that may cause severe ocular inflammation leading to blindness if not treated early. *Acanthamoeba* keratitis (AK), a severe form of corneal infection associated with intense pain has been observed amongst the contact lens wearer population. Contact lens use is one of the important risk factors in contracting Acanthamoeba keratitis. This is of particular concerns for less developed countries where the use of contact lenses is on the rise, with limited awareness of associated risks.

Globally, AK is a cause for concern and an increase in the number of contact lens users with AK is reported with the number of cases gradually increasing since 2005 (Por *et al*. 2009; Joslin *et al*. 2006; Jae *et al*. 2009). In Malaysia, the first case of *Acanthamoeba* keratitis was reported in 1995 involving a woman who was a long-term contact lens wearer (Mohamed Kamel & Norazah 1995). Since then, this condition is no longer a rarity and is seen with increasing frequency especially among contact lens wearers. In 2003, 11 cases of AK were reported from the Universiti Kebangsaan Malaysia Hospital (HUKM) alone (Mohamed Kamel et al. 2018).

The main risk factor for corneal infection in contact lens wearers is the use of contact lens disinfecting systems ineffective at killing *Acanthamoeba* cysts and trophozoites (Tzanetou et al. 2006). The infection is due to contamination of contact lens storage systems, poor contact lens hygiene and ineffective contact lens disinfecting solutions (Gray *et al*. 1995; Schaumberg *et. al* 1998). The cysts and trophozoites attach to the surface of contact lenses and are transmitted to the eye. Thus adequate and appropriate contact lens hygiene and use of effective contact lens disinfecting and storage systems are essential in preventing corneal infection.

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Table 1: Isolation details of *Acanthamoeba* isolates

No.	Strain	Isolate	Source	Sample Type	Morphological group	Place of isolation	Date of isolation
1.	Environmental	TKT19	Soil	Soil sample	Group (II)	Teluk Kemang - Negeri Sembilan	19/4/2012
2.	Environmental	TTT1	Soil	Soil sample	Group (II)	Tanjung Tuan - Negeri Sembilan	1/4/2012

Table 2: Contact lens disinfecting solution Properties

Contact lens Disinfecting solution (CLDS)	Manufacturer	Active ingredient	System Type	Contact Lens Type	Manufacturers Recommendation Soaking Time (Hours)
Oxysept®	Abbott Medical Optics Inc.	Hydrogen Peroxide H_2O_2 (3%)	HPS	All Types	6

Table 3: Efficacy of Oxysept® hydrogen peroxide system (without neutralizing tablet) against *Acanthamoeba* cysts

			Soaking period (hours)		
No.	Strain	Isolate	4hrs.	6* hrs.	8** hrs.
1.	Environmental	TKT19	(-)	(-)	(-)
2.	Environmental	TTT1	(-)	(-)	(-)

(+) Presence of *Acanthamoeba* trophozoites (disinfectant not effective)
(-) Absence of *Acanthamoeba* trophozoites (disinfectant effective)
* Manufacturer recommended soaking time.
** Over night soaking time.

Commercially available contact lens disinfecting solutions are not required to prove its effectiveness against *Acanthamoeba* cysts. The increasing incidence of AK among contact lens wearers has necessitated the evaluation and development of potential therapeutic agents and contact lens disinfectants active against the resistant cyst stage of the organism. Oxysept® hydrogen peroxide system is one of the commonly available and popular contact lens disinfecting solutions in Malaysia and this study was carried out to evaluate its effectiveness as anti-*Acanthamoeba* agent.

MATERIALS AND METHODS

Source of Acanthamoeba

Two environmental isolates of *Acanthamoeba* were used in this study and obtained from the *Acanthamoeba* Lab, Faculty of Medicine, Universiti Kebangsaan Malaysia. The isolates were coded as TTT1 and TKT19 and the isolation details are shown in Table 1.

Contact Lens Disinfecting Solution (CLDS)

The contact lens disinfecting solution used in this study is Oxysept® and its properties are shown in Table 2. The recommended soaking time is 6 hours.

Contact Lens Disinfecting Solution (CLDS) Efficacy Test

The efficacy test for Oxysept® hydrogen peroxide disinfecting system against the *Acanthamoeba* cysts was adopted from method by Narasimhan *et al*. 2002 with some modifications.

The contact lens disinfecting solution efficacy test was carried out using 12-well microtiter plate where 1 ml of the contact lens disinfecting solution was placed in each well. 100 µl of the cyst suspension with an approximate concentration of 1 x 10^5 cyst/ml was pipetted into the wells containing contact lens disinfecting solutions. The cyst suspension was vortexed for 30 seconds before pipetted into each well. The microtiter plates were covered with aluminium foil to prevent drying out and also to mimic the dark conditions of a contact lens storage case. All the microtiter plates were incubated at room temperature following the time parameters as recommended by the manufacturer (6 hours); 4 hours and 8 hours (representing overnight soaking). The contact lens disinfecting solution Oxysept® was tested with and without the neutralizing tablets.

Positive and negative controls were run together with the test samples. Two types of positive controls were run. The first positive control is the cyst suspension in one ml of PAGE saline. The second positive control is the cyst suspension with 3% hydrogen peroxide together with neutralizing tablet. Two types of negative controls were used comprising the Non-Nutrient Agar and cyst suspension with contact lens neutralizer.

Sub-culturing *Acanthamoeba*

Agar plates containing *Acanthamoeba* were sub cultured onto Non-Nutrient Agar seeded with *E. coli* and allowed to grow and encyst for 11 days following the method by Narasimhan *et al*. 2002. The *Acanthamoeba* cyst suspension used in this study was standardized to a concentration of 10^5 cysts per ml using a Neubauer Chamber.

Cyst Morphological Test

The *Acanthamoeba* isolates were morphologically classified by studying the internal structure of the cyst as described by Duarte *et al*. 2013; Pussard & Pons 1977. The distinctive features of the cysts have led to classification into three major groups ie; group I or *Astronyxid*, group II or *Polyphagid* and group III or *Culbertsonid*. The cysts were observed under X100 magnification using ZEISS A1(AX10) compound microscope with LCD attachment and 0.01% Methylene blue dye staining to enhance the morphology of the cyst for better differentiation.

RESULTS

Tables 3-5 show the results of the effectiveness of Oxysept® contact lens disinfecting solution as anti-*Acanthamoeba* agent when tested against the environmental isolates of *Acanthamoeba*. The agar plate that shows the presence of trophozoites after 3 days incubation was recorded as positive. Negative results were recorded for agar plates that did not show the presence of trophozoites after daily observation under inverted microscope for 14 days.

Oxysept® without the addition of neutralising tablet was found to be effective in inactivating the cysts of all *Acanthamoeba* isolates for all the soaking times tested (4, 6 and 8 hours) as shown in Table 3. However when combined with the neutralising tablet simultaneously, as recommended by the manufacturer, Oxysept® fails to exhibit anti-*Acanthamoeba* activity (Table 4). The positive control of *Acanthamoeba* cyst suspension for all isolates showed the presence of trophozoites (Table 4). The Non Nutrient Agar (NNA) media used as a negative control showed no contamination as shown in Table 5. The results for both positive and negative controls were as expected.

The result of cysts morphological test under ZEISS Scope A1(AX10) compound microscope with imaging software shows that all the environmental *Acanthamoeba* isolates are from morphological group II or *Polyphagid*. Its features include small cyst size with diame-

Table 4: Positive control result (Microbiological and Pharmacological)

No.	Strain	Isolate	(Microbiological positive control) Cyst suspension (parasite viability test)	(Pharmacological positive control) Cyst suspension + Oxysept® "3% H_2O_2 + neutralizer tablets" (HP system viability test)			
				4hrs.	*6hrs.	**8 hrs.	14 days
1.	Environmental	TTT1	(+)	(+)	(+)	(+)	(+)
2.	Environmental	TKT19	(+)	(+)	(+)	(+)	(+)

(+) Presence of *Acanthamoeba* trophozoites in the media after incubation for 3 days (Positive microbiological control) and Presence of *Acanthamoeba* trophozoites after daily observation for 14 days (Positive pharmacological control).

(-) Absence of *Acanthamoeba* trophozoites in the media after incubation for 3 days (Positive microbiological control) and Absence of *Acanthamoeba* trophozoites after daily observation for 14 days (Positive pharmacological control).

* Manufacturer recommended soaking time.

** Over night soaking time.

Table 5: Negative control result (Microbiological and Pharmacological)

No.	(Microbiological negative control) NNA media (Contamination test)	Isolate	(Pharmacological negative control) cyst suspension + Oxysept® "neutralizer tablets only" (HP system viability test)			
			4 hrs.	*6 hrs.	**8 hrs.	14 days
1.	(-)	TTT1	(+)	(+)	(+)	(+)
2.	(-)	TKT19	(+)	(+)	(+)	(+)

(+) Presence of bacterial or fungal colonies growth in NNA media after incubation for 3 days (Negative microbiological control) and Presence of *Acanthamoeba* trophozoites after daily observation for 14 days (Negative pharmacological control).

(-) Absence of *Acanthameba* trophozoites in NNA media after incubation for 3 days (Negative pharmacological control) and Absence of *Acanthamoeba* trophozoites after daily observation for 14 days (Negative pharmacological control)

* Manufacturer recommended soaking time.

** Over night soaking time.

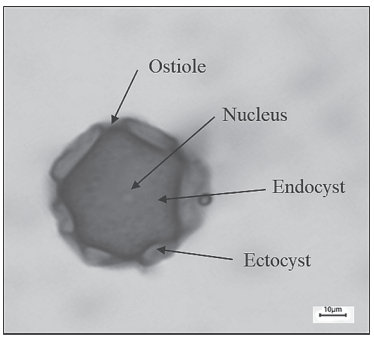

Figure 1: Morphological group classification of *Acanthamoeba* cyst using 0.01% Methylene Blue Stain

ter lesser than 18 µm, irregularly wrinkled ectocyst arches and wide polygonal endocyst as shown in Figure 1.

DISCUSSION

Acanthamoeba keratitis was extremely rare before the introduction of contact lenses. The increasing incidence of *Acanthamoeba* keratitis among contact lens wearers makes it advisable to use effective contact lens disinfecting systems aimed at killing *Acanthamoeba* cysts and trophozoites, as well as bacteria and fungi (Tzanetou *et al*. 2006). The main target for prevention of corneal infection is to keep the contact lenses and contact lens storage cases free of viable *Acanthamoeba* and microorganisms. The hydrogen peroxide disinfectants, which destroy the pathogens by oxidation, are available in two systems: one-step hydrogen peroxide that has no separate neutralization step and two-step hydrogen peroxide which, after a recommended disinfection time, requires a second neutralization step. The contact lens disinfecting systems used in this study was the one-step 3% hydrogen peroxide solution.

The results of this study indicate that disinfecting system using hydrogen peroxide without the simultaneous addition of the neutralising tablet is effective in inactivating *Acanthamoeba* cysts. This is consistent with the findings of our previous study done in 2019 but using cysts from clinical *Acanthamoeba* isolates (Mohamed Kamel *et al*. 2019). Similar findings have also been reported by Johnston *et al*. (2009), Hughes & Kilvington (2001), as well as Hiti *et al*. (2006). A study by Shoff *et al*. (2007) found varying sensitivities between environmental isolates of Acanthamoeba towards contact lens disinfecting solutions.

When Oxysept® is combined with the neutralising tablet simultaneously, as recommended by the manufacturer, Oxysept® fails to exhibit anti-*Acanthamoeba* activity at all soaking times. The disinfecting ability of hydrogen peroxide is directly proportional to the time of exposure of the organisms to the active ingredients of the solution. For neutralization, the one-step 3% hydrogen peroxide uses a neutralizer catalase tablet (Oxysept 1 Step) from the very beginning of the disinfection step, thus the decomposition of hydrogen peroxide into water and oxygen occurs very early, before disinfection occurs (Tzanetou *et al*. 2006). This renders failure in inactivating *Acanthamoeba* cysts.

Hughes and Kilvington (2001) found that two-step hydrogen peroxide disinfecting system is more effective in inactivating *Acanthamoeba* cysts compared to one-step hydrogen peroxide disinfecting system. They also found that at least 6 hours is required to kill all *Acanthamoeba* cysts. The amoebicidal activity of one-step hydrogen peroxide could be improved if the neutralization rate was delayed. Otherwise the parasite would remain viable on the contact lens surface after soaking in one-step 3% hydrogen peroxide disinfecting solution (Tzanetou et al. 2006). Reduced efficacy of commercial one-step 3% hydrogen peroxide against the strains *Acanthamoeba polyphaga* (Hughes & Kilvington 2001) *Acanthamoeba castellanii*, *Acanthamoeba hatchetti* and *Acanthamoeba lenticulata* (Hiti *et al*. 2002) has also been reported.

CONCLUSIONS

Oxysept® hydrogen peroxide disinfecting solution would only exhibit anti-*Acanthamoeba* activity if it is used without simultaneously adding the neutralizing tablets. The amoebicidal activity of one-step hydrogen peroxide disinfectant could be improved if the neutralization process is delayed.

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