OPHTHALMOLOGY

Susceptibility of Environmentally Isolated *Acanthamoeba* to Chlorhexidine and Propamidine Isethionate

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ABSTRACT

Introduction: ***Acanthamoeba*** **keratitis (AK) is becoming an increasingly well-known clinical entity, resulting in corneal infections that are refractory to medical therapy. The** ***Acanthamoeba*** **species in their encysted state are resistant to antimicrobial agents and these render medical treatment difficult.**

Objective: **This in-vitro susceptibility testing study was conducted to determine the effectiveness and the minimum cysticidal concentration (MCC) of 0.02% Chlorhexidine and 0.1% Propamidine isethionate (Brolene®) against four environmental isolates of** ***Acanthamoeba*** **ie; PHS 11, PHS 15, TLA 1 & KSA 13.**

Materials and Methods: **The in-vitro susceptibility test adopted the method by Narasimhan** ***et al.*** **with slight modification. Briefly, serial doubling dilutions of the antimicrobial agents were performed in microtiter plates. After exposure of the** ***Acanthamoeba*** **cysts to the antimicrobial agents for 24 hours, the cysts were washed three times with PAS and centrifuged. The deposits (cysts) were cultured onto non-nutrient agar coated with heat-killed** ***Escherichia coli.*** **The excystment of trophozoites from cysts was observed and recorded microscopically for 14 days to determine the MCC value of each drug.**

Result: **Chlorhexidine successfully exhibited cysticidal activities on all isolates while propamidine was effective on the majority of the isolates. The mean MCC values of Chlorhexidine and Propamidine were 10.9 µg/ml and 296.8 µg/ml respectively.**

Conclusion: **Both Chlorhexidine and Propamidine are effective anti-*Acanthamoeba* agents and the combination is still suitable for the treatment of** ***Acanthamoeba*** **keratitis.**

KEY WORDS

Acanthamoeba**, in vitro susceptibility test, minimum cysticidal concentration, Malaysia**

INTRODUCTION

Acanthamoeba is a free living amoeba capable of causing severe eye, brain and skin infections. It is widely distributed in the environment, occurring in vegetative trophozoite, and a double-walled cyst. Trophozoite is the active form that feeds on bacteria and yeast and encysted into a resistance cyst under unfavourable environmental conditions such as nutrients deficiency, extremes of temperature, and pH changes[1)]. In the environment, *Acanthamoeba* has been isolated from the soil, water, air, birds, animals and medical instrumentation[2,3)].

Acanthamoeba keratitis (AK) is a painful sight-threatening disease of the eyes which may lead to blindness and may require corneal transplantation to repair the eye damage[4)]. In serious cases when the amoebae have invaded tissues beneath the cornea, enucleation of the eye has been necessary to provide emergency relief for patients. *Acanthamoeba* keratitis occurs worldwide and many AK outbreaks in both developed and developing countries have been reported[5)]. The development of AK has been mostly linked to contact lens use[6,7)] and also to the exposure to contaminated soil or water following trauma[8)].

In Malaysia, many cases of AK have been reported and individuals with AK have been treated successfully with a combination of chlorhexidine digluconate and propamidine isethionate[9-11)]. However, there is still a need to test the current antimicrobial agents such as chlorhexidine and propamidine since previous studies have reported that some strains of *Acanthamoeba* are already resistant to these agents[12,13)]. Hence, the present study aims to determine the effectiveness and the minimum cysticidal concentration (MCC) of the two antimicrobial agents in therapeutic dose against four local environmental isolates of *Acanthamoeba*. This exercise would contribute to the useful monitoring or surveillance for the susceptibility patterns of *Acanthamoeba* isolates in Malaysia.

MATERIALS AND METHODS

Acanthamoeba Isolates

Four *Acanthamoeba* environmental isolates namely PHS 11, PHS

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Table 1. Isolation details of *Acanthamoeba* isolates.

Code	Source	Location of isolation
PHS 11	Tap water	Poring Hot Spring Sabah
PHS 15	Tap water	Poring Hot Spring Sabah
TLA 1	Sea water	Marine Park Tioman Pahang
KSA 13	Sea water	Marine Park Tioman Pahang

Table 3. MCC of chlorhexidine and propamidine against *Acanthamoeba* isolates.

Acanthamoeba Isolates	Antimicrobial agents MCC (µg/ml)	
	Chlorhexidine	Propamidine isethionate
PHS 11	6.25	1000
PHS 15	25.0	62.5
TLA 1	6.25	Ineffective
KSA 13	6.25	125
Overall mean	10.9	296.8

15, TLA 1 & KSA 13 were used in this study and the isolation details are shown in Table 1.

Acanthamoeba cyst age and their standard suspension

Cyst suspension used throughout this study consisted only of *Acanthamoeba* cysts that have reached maturity at the age of 14 days of culture on non-nutrients agar (NNA). An agar plate containing many *Acanthameoba* cysts and free from contamination was observed under inverted microscope. One ml solution of the Page saline was added onto the agar surface and the *Acanthamoeba* cysts were harvested by using a wire loop. The cyst concentration was adjusted to be standardized at 1 x 10^5 cysts per ml. Calculation of cyst concentration was done by using a Neubauer Chamber.

Antimicrobial Agents Used in the in Vitro Susceptibility Test

0.02% chlorhexidine digluconate (SIGMA, Spain) and 0.1% propamidine isethionate (Brolene®) (Sanofi-aventis, Ireland) were used in this study.

Dilutions of the Antimicrobial Agents

Serial doubling dilution was performed in 96 wells microtiter plate with U-shaped wells which were labelled from 1 to 14. Two negative controls were used in the 13th and 14th wells, and other two positive controls were prepared outside the microtiter plate in two eppendorf tubes.

The serial doubling dilution of chlorhexidine ranged from 200 µg/ml to 0.0977 µg/ml and propamidine (Brolene®) ranged from 1000 µg/ml to 0.4883 µg/ml; solutions were prepared using Page saline solution. The microtiter plate, together with the two eppendorf tubes, were sealed and incubated at 30°C for 24 hours. Each test was performed in duplicate.

Evaluation of Effectiveness of the Antimicrobial Agents

Chlorhexidine and propamidine solutions were directly exposed to the four *Acanthamoeba* isolates in the 96 wells microtiter plate using working stock of 200 µg/ml and 1000 µg/ml respectively.

Determination of the minimum cysticidal concentration (MCC) of antimicrobial agents

The method described by Narasimhan[14] *et al*. 2002 was modified to be used in this susceptibility test which involved the used of micro-dilution technique for the antimicrobial agents and monoxenic culture for the *Acanthamoeba* isolates. After 24 hours incubation at 30°C, the wells mixture were rinsed three times with 100 µl Page saline solution in eppendorf tube to ensure no cysts were left behind the wells and to rinse off the antimicrobial agents surrounding the cysts.

Table 2. Effectiveness of Antimicrobial agents in therapeutic dose against *Acanthamoeba* cysts.

Isolates	0.02% Chlorhexidine digluconate	0.1 % Propamidine isethionate
PHS 11	✓	✓
PHS 15	✓	✓
TLA 1	✓	X
KSA 13	✓	✓

Key:

✓ Effective (trophozoites absent)

X Ineffective (trophozoites present)

After centrifuging the eppendorf tubes, the sediment (cyst) was transferred to the NNA coated with heat-killed *Escherichia coli* suspension and incubated for 48 hours at a temperature of 30°C. Then, after 48 hours of incubation, the plates were observed under the inverted microscope to detect the presence of trophozoites that emerge from the cysts. Observation was done for 14 days before confirming negative result.

MCC was defined as the lowest concentration of the test solution (an antimicrobial agent) that resulted in no excystment and growth of trophozoite of *Acanthamoeba* from cysts after 14 days of incubation. The results were compared with the positive control which should show the appearance of trophozoites within 24 to 48 hours incubation on the inoculated NNA coated with *Escherichia coli* on the surface plates.

Methylene Blue 0.01% w/v Preparation

Methylene blue powder of 0.01g was dissolved in 10 ml of distilled water to provide 0.1 % w/v methylene blue solution. Then, 1 ml of 0.1% w/v of methylene blue was dissolved in 9 ml of distilled water and vortexed for a minute. Thus, the solution of 0.01% w/v methylene blue was available and stored at room temperature.

Statistical Analysis

Data analysis was performed by using the SPSS software version 13.0 for windows (SPSS, Chicago, IL. USA). Mean MCC values of the antimicrobial agents were compared by using one way ANOVA. A p value ≤ 0.05 was considered statistically significant.

RESULTS

Chlorhexidine exhibits cysticidal activities against all four environmental isolates of *Acanthamoeba* at therapeutic doses while Propamidine with only 3 isolates (Table 2) and the MCC results are shown in Table 3. Propamidine fails to inactivate cysts isolate TLA 1.

Serial doubling dilution for chlorhexidine was performed with concentrations ranging from 200 µg/ml to 0.0977 µg/ml. For propamidine isethionate (Brolene®), serial doubling dilution was performed with concentration ranging from 1000 µg/ml to 0.4883 µg/ml and the mean MCC value was 296.8 µg/ml. However, chlorhexidine showed a lower mean MCC value compared to propamidine. The MCC mean value of chlorhexidine was 10.9 µg/ml in which the MCC obtained from this study ranged from 6.25 to 25 µg/ml.

The difference in MCC between the drugs was found to be significant (ANOVA; $P < 0.001$). The output of ANOVA test revealed that chlorhexidine showed the highest effectiveness compared to propamidine. Both positive and negative controls performed as expected.

Cyst Groups Identification

In this study, the *Acanthamoeba* environmental isolates cysts have been assigned into three groups (I, II and III) according to their morphology (size and shape) as described by Pussard & Pons[15] and following methylene blue staining under inverted microscope (Fig. 1). Based on their cysts morphology, PHS 15 has been assigned to group I (Astronyxids), PHS 11 to group II (Polyphagids) while TLA 1 and KSA 13 to group III (Culbertsonids).

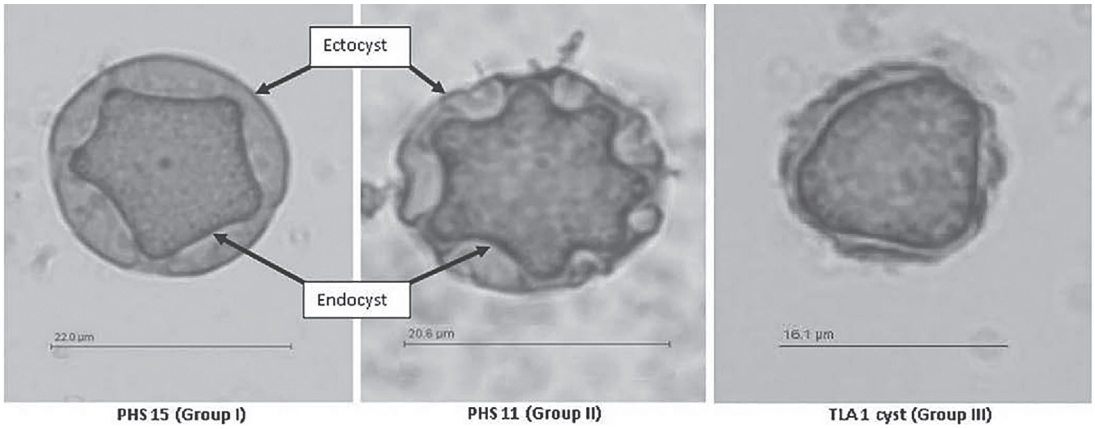

Figure 1. ***Acanthamoeba*** **cyst stage group I, II, III (phase contrast 1000 x).**

DISCUSSION

Drugs that are considered effective against *Acanthamoeba* cysts must penetrate the cyst wall and act on the internalized amoeba. *Acanthamoeba* cysts, which have been reported to be highly resistant to antimicrobial and antiparasitic drugs as well as environmental factors, can maintain their viability and virulence for up to 25 years[16,17]. In this study, 0.02% chlorhexidine, showed the best cysticidal activity with the lowest mean MCC against all four *Acanthamoeba* isolates at minimal concentrations that are not toxic to corneal epithelial cells which can be tolerated by the eyes. It is considered as the first line of medical therapy either alone or in combination to avoid the occurrence of resistance in the treatment of AK[18]. This is consistent with the findings by Shirley[19] *et al*. 2011 and against clinical isolates, chlorhexidine was equally sensitive but with a slightly higher MCC value as we previously reported in 2016[20]. Similar findings were also reported by other previous studies[21,22].

Chlorhexidine may act by binding its highly charged positive molecules to the mucopolysaccharide in the *Acanthamoeba* cyst wall plug of the ostiole. This may lead in penetration through the ostiole to the internalized amoeba, where the drug binds to the phospholipid bilayer of the amoeba cell membrane causing membrane damage, cell lysis and death[23,24].

Propamidine isethionate (Brolene®) also exhibited cysticidal activity against majority of *Acanthamoeba* isolates used in the current study except for isolate TLA 1 which was resistant to propamidine. This findings are similar to a report by Shirley[19] *et al*. 2011. Even when tested against clinical isolates, propamidine remains effective (Mohamed Kamel *et al*. 2016)[20]. In Great Britain, many cases of AK have been successfully treated with propamidine which is available as over the counter eye medication[25]. Its effectiveness might be due to the ability to bind and penetrate the *Acanthamoeba* cell membrane or as DNA synthesis inhibitor[26]. Moreover, it can also inhibit oxygen uptake and induce leakage of amino acids[27].

The development of propamidine resistance was reported by Ficker *et al*., during the course of treatment for AK as a monotherapy[12]. However, that development of resistance could be through inhibition of polyamines synthesis of *Acanthamoeba* which leads to block the induction of encystment, but may not prevent the arrest of cell multiplication by these inhibitors which may induce resistance[28].

Important factors which may influence the in-vitro susceptibility testing involve the maintaining of cyst number against antimicrobial eye drop and the geographical distribution of *Acanthamoeba* strains[6,14]. Moreover, culturing conditions, kind of *Acanthamoeba* isolates, cysts storage time, procedures of drugs susceptibility test may also result in slight variations in MCC results[23]. In the present study, 2 isolates belong to group III (Culbertsonids) while the rest, each belongs to group I (Astronyxids) and group II (Polyphagids). A previous study by Walochnik[29] *et al*. 2000, reported that *Acanthamoeba* group II (Polyphagids) was the most prevalent among all the samples collected, followed by group III. Despite that, we found TLA 1 from group I (Astronyxids) being more resistant than other isolates.

CONCLUSION

In conclusion, this study reveals that 0.02% Chlorhexidine, which is considered as a first line treatment of AK, still demonstrating the best cysticidal activity. Chlorhexidine was reported as the most effective antimicrobial agent against *Acanthamoeba* either in vitro or in vivo studies, and it can be used as monotherapy or in combination with propamidine. Our findings did not encourage the use of 0.1% propamidine as monotherapy in the treatment of AK. However, it might be used in combination with other antimicrobial agents that are not toxic to the eye. Hence, the in-vitro susceptibility testing of antimicrobial agents would be a useful guide for ophthalmologists to decide on whether to continue or discontinue therapy with specific drugs.

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