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In Vitro Sensitivity Testing of *Acanthamoeba* Clinical Isolates from Patients with Keratitis against Polyhexamethylene biguanide (PHMB) and Chlorhexidine

(Kajian Sensitiviti Isolat Klinikal *Acanthamoeba* daripada Pesakit Keratitis Secara *In Vitro* Terhadap Polyhexamethylene biguanide (PHMB) dan Chlorhexidine)

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ABSTRACT

Acanthamoeba *keratitis is a serious infection of the eye which can result in permanent visual impairment. Therefore this study was performed to evaluate the effectiveness of antimicrobial agents on three* Acanthamoeba *clinical isolates (HS 6, HKL 95 and HTH 73). Antimicrobial agents used in this study were polyhexamethylene biguanide (PHMB) and chlorhexidine and both were serially diluted. Cyst suspensions from all three strains were tested against the antimicrobial agents, respectively. After 48 h of incubation at 37°C, the suspension was filtered and the filter membrane was placed onto non-nutrient agar plate lawned with heat-killed* Escherichia coli. *The plates were examined daily under the inverted microscope until day 14 but were negative for* Acanthamoeba *trophozoites. The presence of trophozoites indicated ineffectiveness of the antimicrobial agents. Both antimicrobial agents used were found to be effective against* Acanthamoeba *cysts for all the strains tested. PHMB gave minimum cysticidal concentration (MCC) mean value of 4.232 µg/mL and chlorhexidine showed MCC mean value of 3.906 µg/mL. So, from this study, it can be concluded that PHMB and chlorhexidine were effective in killing the tested* Acanthamoeba *cysts.*

Keywords: Acanthamoeba*; in vitro; sensitivity; polyhexamethylene biguanide; chlorhexidine*

ABSTRAK

Keratitis Acanthamoeba *merupakan infeksi mata yang serius yang boleh menyebabkan kerosakan penglihatan yang teruk. Oleh yang demikian, kajian ini dilakukan untuk mengkaji keberkesanan agen antimikrob terhadap tiga isolat klinikal Acanthamoeba (HS 6, HKL 95 and HTH 73). Agen antimikrob yang digunakan di dalam kajian ini adalah* poliheksametilina *biguanide (PHMB) dan* kloroheksidin *yang menjalani pencairan bersiri. Suspensi sista daripada ketiga-tiga strain diuji dengan kedua-dua agen antimikrob. Selepas inkubasi selama 48 jam pada suhu 37°C, campuran suspensi sista dan agen antimikrob tersebut dituras dan membran turasan diletakkan ke atas plat agar bukan nutrien yang dibanjiri* Escherichia coli. *Kesemua agar tersebut diperiksa setiap hari menggunakan mikroskop terbalik sehingga hari ke 14 tetapi negatif untuk trofozoit* Acanthamoeba. *Kehadiran trofozoit menunjukkan ketidakberkesanan agen antimikrob. Kedua-dua agen antimikrob yang digunakan didapati berkesan terhadap sista* Acanthamoeba *bagi kesemua strain yang diuji. PHMB memberikan nilai MCC pada kepekatan 4.232 µg/mL manakala chlorhexidine pula menunjukkan nilai MCC pada kepekatan 3.906 µg/mL. Maka, daripada kajian ini, dapat disimpulkan bahawa PHMB dan* kloroheksidina *berkesan membunuh sista* Acanthamoeba *yang diuji.*

Kata kunci: Acanthamoeba; *in vitro; kloroheksidine; poliheksametilena biguanide; sensitivitc*

INTRODUCTION

Acanthamoeba spp. are ubiquitous in aquatic habitats and colonize biofilms in swimming pools, hot tubs, domestic water taps, eyewash stations, contact lens disinfecting solutions, and air-conditioning systems in buildings and automobiles (Simmons et al. 1999). The trophozoite is the active motile stage that feeds by phagocytosis and pinocytosis and divides by binary fission. A cyst stage is resistant to adverse environmental conditions such as desiccation, extreme temperatures and antimicrobials (Borazjani et al. 2000).

Acanthamoeba keratitis (AK) is a rare but sight-threatening ocular infection caused by *Acanthamoeba*. Outbreaks of AK have been associated with contaminated water and contact lens wear (Ledee et al. 2009). In recent years, the number of AK cases has been on the increase, especially among wearers of contact lenses, who make up 85 to 90% of the AK cases (Patel & Hammersmith 2008). In Malaysia, *Acanthamoeba* keratitis is not so rare and the incidence has increased as reported between June and December 2002, four (9.1%) of 44 cases were found to be

culture positive for *Acanthamoeba* (Kamel et al. 2005). AK can be the primary infection or present as a suprainfection in combination with other infectious organisms, like bacteria or fungi, complicating diagnosis and treatment. Diagnosis of AK is problematic due to clinical features which are similar to those of herpetic, bacterial, and fungal infections (Goodall et al. 1996).

The encystment capability of *Acanthamoeba* species confounds treatment due to the recalcitrant nature of the cyst to most treatment options allowing reemergence of amoebae after treatment cessation (Ledee et al. 2009). *Acanthamoeba* keratitis is considered as one of the most difficult ocular infections to manage successfully due to the resistance of the cyst stage of the organism to most antimicrobial agents at concentrations tolerated by the cornea (Kilvington et al. 2002). The *in vitro* studies of susceptibility of *Acanthamoeba* spp. can be used as a standard test for assay of MCC of drugs on *Acanthamoeba* isolates and to study the susceptibility pattern of newer water-soluble anti-*Acanthamoeba* drugs. This study was conducted to determine the effectiveness and MCCs of the tested drugs on the local clinical isolates due to their promising therapeutic effects on *Acanthamoeba* keratitis.

Materials and Methods

Source of *Acanthamoeba*

Acanthamoeba isolates were obtained from the *Acanthamoeba* Culture Laboratory, Universiti Kebangsaan Malaysia, subcultured from clinical specimens from local hospitals in Malaysia (Hospital Selayang, Hospital Kuala Lumpur and the Tun Hussein Onn National Eye Hospital).

Source of Antimicrobial Agents

The antimicrobial agents tested towards *Acanthamoeba* cyst were polyhexamethylene biguanide (PHMB) and chlorhexidine which were obtained from the Tun Hussein Onn National Eye Hospital. Both antimicrobial agents were used before the expiratory dates.

Sensitivity Test

Acanthamoeba cyst suspension was prepared and vortexed for about one minute to ensure the cysts were well mixed in the Page Amoebic Saline solution. Essentially, double dilutions of each antimicrobial agent were performed in microtitre plates with five uL cysts at the concentration of 1×10^5 organisms per 100 µL of medium per well. Microtitre plates were incubated at 37°C for 48 h (Narasimhan et al. 2001).

Two positive controls were prepared with cysts mixed with Page Amoebic Saline solution and the other with cysts mixed with 30% hydrogen peroxide. Two negative controls on the other hand were prepared with only the antimicrobial agents (polyhexamethylene biguanide (PHMB) and chlorhexidine) without the addition of the cyst. The sensitivity testing were done in duplicates.

The mixture of the antimicrobial agents (PHMB and chlorhexidine) and *Acanthamoeba* cyst respectively, positive and negative controls were filtered using the filtration unit which consists of millipore, vacuum pump and nitrate cellulose membrane as the main components. The nitrate cellulose membrane used measures 0.45 µm in pore size. Each microtiter well was rinsed using Page Amoebic Saline solution to detach any remaining cyst. Rinsing was done twice. After filtration, the nitrate cellulose membrane was placed onto the non-nutrient agar plates seeded with heat-killed *Escherichia coli* as the source of food for *Acanthamoeba*. The agar plate was then incubated at 37°C for three days.

After three days, the membrane was removed from the agar plate. Each agar plate was examined daily under the inverted microscope for the presence of trophozoite. Observation was done daily until day 14 to confirm the result before the plate was discarded.

In this study, the focus was given to *Acanthamoeba* cysts as they are more resistant against any treatment or antimicrobial agents exposed to them. The lowest antimicrobial concentration preventing trophozoites formation after 14 days incubation was taken as the minimum cysticidal concentration (MCC).

Results

Polyhexamethylene Biguanide (PHMB) and Chlorhexidine in Vitro Sensitivity Test Against Acanthamoeba Cysts

In this study, it was found that both antimicrobial agents used were effective when exposed to all the clinical test strains used. The minimum cysticidal concentration (MCC) value for polyhexamethylene biguanide tested against HS 6, HKL 95 and HTH 73 are shown in Table 1. In this study, *Acanthamoeba* HS 6 strain showed sensitivity towards PHMB with the MCC of 7.813 µg/mL during both first and second tests. The MCC values obtained when HS 6 was exposed to chlorhexidine were 7.813 µg/mL in the first test and 3.906 µg/mL in the second test, giving the mean MCC value of 5.859 µg/mL.

With the exposure to PHMB at 37ºC for 48 h, HKL 95 strain showed sensitivity at the MCC value of 1.953 µg/mL during first and second tests. Meanwhile, when exposed to chlorhexidine, MCC value obtained were 1.953 µg/mL in the first test and 3.906 µg/mL in the second test, with the mean MCC value of 2.930 µg/mL. The MCC value of 1.953 µg/mL during the first test and 3.906 µg/mL during the second test were obtained when HTH 73 was exposed to PHMB. The mean MCC value obtained for both test was 2.930 µg/mL. On the other hand, when HTH 73 was exposed to chlorhexidine, MCC value obtained were 1.953 µg/mL in the first test and 3.906 µg/mL in the second test, with mean MCC value of 2.930 µg/mL.

TABLE 1. Minimum cysticidal concentration (MCC) value for polyhexamethylene biguanide (PHMB) and chlorhexidine tested against *Acanthamoeba* isolates from Patients with Keratitis

Isolates of *Acanthamoeba*	MCC (μg/mL)					
	Polyhexamethylene biguanide			Chlorhexidine		
	First test	Second test	Mean	First test	Second test	Mean
HS 6	7.813	7.813	7.813	7.813	3.906	5.859
HKL 95	1.953	1.953	1.953	1.953	3.906	2.930
HTH 73	1.953	3.960	2.930	1.953	3.906	2.930
MCC mean			4.232			3.906

TABLE 2. Results of the positive and negative controls for the in vitro sensitivity testing of *Acanthamoeba* clinical isolates against PHMB and chlorhexidine

Isolates of Acanthamoeba	Control			
	Positive		Negative	
	30% H_2O_2	PAS	PHMB	Chlorhexidine
HS 6	–	+	/	/
HKL 95	–	+	/	/
HTH 73	–	+	/	/

Indicator :

+	Presence of cyst and trophozoite	PAS	Page amoebic saline
-	Absence of trophozoite	H_2O_2	Hydrogen peroxide
/	Absence of cyst and trophozoite	PHMB	Polyhexamethylene biguanide

POSITIVE AND NEGATIVE CONTROLS

Positive controls were prepared with cysts exposed to hydrogen peroxide and Page amoebic saline, respectively. No trophozoite was observed until day 14 when the cysts were exposed to hydrogen peroxide as seen in Table 2. Meanwhile, trophozoites were seen in the plate containing cysts added with Page amoebic saline.

Negative controls were prepared with the antimicrobial agents alone without the addition of cyst to ensure the sterility of the antimicrobial agents from any contamination. As seen in Table 2, no cyst and trophozoite were observed until day 14 for the negative controls.

RESULTS AND DISCUSSION

Polyhexamethylene biguanide (PHMB) was initially used as a pool disinfectant and it has a broad activity towards amoeba and bacteria. Its bactericidal activity is through interrupting the bacteria cell wall causing leakage of the cell wall and followed by cell death. Meanwhile, chlorhexidine is classified as a disinfectant or a detergent. At a lower concentration, it has been used in the treatment of diseases related to the skin, ear and oral cavity (Watson 2002).

Among many antimicrobials tested against *Acanthamoeba* spp., the biguanides, PHMB and chlorhexidine digluconate appear to be the most effective. Various multipurpose contact lens solutions include PHMB at concentrations from 0.5 (0.00005%) to 5 μg/mL (0.0005%) (Larkin et al. 1992). These concentrations are significantly lower than the MCC of PHMB necessary to kill 10^5 cysts of *Acanthamoeba* spp. per ml. Minimal amoebicidal concentrations of PHMB and chlorhexidine for 10^5 trophozoites/ml range from 50 to 100 μg/mL after 24 h of exposure and are as low as 25 μg/mL after 72 h of exposure (Elder et al. 1994).

Lee in his study to evaluate the PHMB cysticidal effect on *Acanthamoeba* cyst found the MCC value at the concentration of 2.37 μg/mL (Lee et al. 1998). In a sensitivity testing of *Acanthamoeba* against PHMB by Kilvington, the mean MCC value obtained was 3.2 ± 0.5 μg/mL at the temperature of 32°C (Kilvington et al. 2002). Lee in his study also obtained 7.02 μg/mL as the MCC value of chlorhexidine towards *Acanthamoeba* cyst (Lee et al. 1998). The mean MCC value of Chlorhexidine in a study done by Kilvington et al. (2002) was 26.7 ± 17.4 μg/mL and Perez Santoja was 2.38 ± 1 μg/mL (Kilvington et al. 2002; Perez-Santoja et al. 2003).

The mean MCC values obtained in this study were more or less similar with the previous studies done by the other researchers. In this study, when *Acanthamoeba* cysts were exposed to PHMB and chlorhexidine, the MCC mean values obtained were 4.232 μg/mL and 3.906 μg/mL, respectively. The MCC mean value of chlorhexidine against *Acanthamoeba* cysts obtained was lower than the studies, by Kilvington et al. and Elder et al. (1994) The MCC value of PHMB obtained in this study was also lower than the MCC value obtained by Elder et al. (1994). This may be

attributed to the different strains used whereby the clinical isolates used were local strains. The specimens which were isolated from the eyes of the patients might not be exposed to drugs prior to the isolation of the *Acanthamoeba*.

Conclusion

From this study, it can be concluded that the clinical isolates of *Acanthamoeba* cysts were sensitive towards polyhexamethylene biguanide (PHMB) and chlorhexidine with the minimum cysticidal concentration of 4.232 µg/mL and 3.906 µg/mL, respectively. Further investigations can be done to test the sensitivity of *Acanthamoeba* of different strains towards different antimicrobial agents.

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