In-Vitro Study of the Cysticidal Effect of Antimicrobial Agents on Acanthamoeba spp. from Clinical Isolates

Mohamed Kamel Abd Ghani1), Siti Ainsyah Mohd Radzi1), Anisah Nordin2), Noraina Ab Rahim2), Vayithiswary Kannan1)

ABSTRACT

Introduction: Acanthamoeba keratitis (AK) is a type of serious corneal infection that may result in severe inflammatory reaction and visual loss. The resistance of Acanthamoeba cysts towards antimicrobial agents has increased from time to time.

Objective: This study was performed to evaluate the effectiveness of the therapeutic dose and the minimum cysticidal concentration (MCC) of 2 antimicrobial agents; 0.02% chlorhexidine digluconate and 0.01% propamidine isethionate on clinical isolates of Acanthamoeba (HUKM 74 and HS 62).

Methods: Serial doubling dilution for chlorhexidine digluconate from 200 to 0.0977 μg/ml and propamidine isethionate from 1000 to 0.4883 μg/ml were performed to obtain their MCC. After exposure of the cysts to each concentration of the antimicrobial agents for 48 hours, the cysts were then cultured onto non-nutrient agar plates overlaid with Escherichia coli. The effectiveness of the therapeutic dose without doubling dilution was determined based on the presence of the trophozoites from the cysts after incubation period. The excystment of trophozoites from cysts was observed and recorded microscopically for 14 days to determine the MCC value of each drug.

Results: Chlorhexidine digluconate and propamidine isethionate were successfully found to be effective against all isolates of Acanthamoeba cysts at therapeutic dose. The mean MCC values for chlorhexidine and propamidine isethionate were 50.0 μg/ml and 312.5 ± 62.5 μg/ml respectively.

Conclusion: Chlorhexidine and propamidine isethionate are effective against all isolates tested and recommended for the treatment of AK.

KEY WORDS

Acanthamoeba, in vitro-sensitivity test, chlorhexidine digluconate, propamidine isethionate

INTRODUCTION

Acanthamoeba is a ubiquitous free living amoeba that can be found widely in nature such as from air, sea water, fresh water, soil, air conditioning units, dialysis units, and eye wash solution1). Acanthamoeba exists in two stages during its life cycle which are vegetative trophozoite stage and a resistant cyst stage. Under favourable conditions, Acanthamoeba remains in the trophozoite form and divides mitotically thus causing infection while under harsh conditions, amoeba transforms into a dormant cyst form that is highly resistant. Since 1960, Acanthamoeba was known to be as a pathogenic organism to humans. Infection by Acanthamoeba parasites is rare but once infected, humans can have severe infection of the eyes and central nervous system2). They have the potential to cause two types of diseases namely Acanthamoeba Keratitis (AK) and Granulomatous Amebic Encephalitis (GAE)3). Acanthamoeba keratitis has been recognized as a significant ocular parasitic infection. The first case of AK was reported in 1974 by Nagington et al. (1974)4). In Malaysia, the first Acanthamoeba keratitis case was reported involving a female contact lens wearer5). In 2001, 10 cases were diagnosed at Hospital Universiti Kebangsaan Malaysia (HUKM) alone and in 2003 another 11 new cases6) were confirmed indicating that Acanthamoeba keratitis is no longer a rarity in Malaysia7). AK can be characterized as pain and redness in the eyes, epithelial lesions, edema and photophobia. In terms of diagnosis, AK is often misdiagnosed as a herpes simplex virus keratitis8). The treatment modalities of Acanthamoeba is limited and becomes a major problem when resistance is encountered9). In vitro susceptibility testing of isolates may prove beneficial for application of early treatment regimens. Thus, this study was conducted to determine the effectiveness and the MCCs of the tested drugs on the clinical isolates by in vitro susceptibility test.

MATERIALS AND METHODS

Acanthamoeba strains

Two clinical isolates (HUKM 74 and HS 62) of Acanthamoeba were obtained from the Acanthamoeba Culture Laboratory, Department of Parasitology, Faculty of Medicine, Universiti Kebangsaan Malaysia. The isolation details are shown in Table 1.
Table 1. Isolation details of *Acanthamoeba* isolates.

<table>
<thead>
<tr>
<th>Isolate code</th>
<th>Date of isolation</th>
<th>Source</th>
<th>Location of isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>HUKM 74</td>
<td>21st February 2017</td>
<td>Keratitis</td>
<td>Hospital Universiti Kebangsaan</td>
</tr>
<tr>
<td>HS 62</td>
<td>31st January 2013</td>
<td>Keratitis</td>
<td>Private hospital in Malaysia</td>
</tr>
</tbody>
</table>

Table 2. The effectiveness test of 0.1% propamidine isethionate and 0.02 % chlorhexidine digluconate in therapeutic dose against *Acanthamoeba* spp. cysts.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>0.1 % propamidine isethionate</th>
<th>0.02 % chlorhexidine digluconate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MCC (μg/ml)</td>
<td>MCC (μg/ml)</td>
</tr>
<tr>
<td>HUKM 74</td>
<td>250.00 250.00 50.00 50.00 50.00</td>
<td></td>
</tr>
<tr>
<td>HS 62</td>
<td>250.00 500.00 375.00 50.00 50.00</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>312.50</td>
<td>50.00</td>
</tr>
</tbody>
</table>

Antimicrobial agents

The antimicrobial agents tested towards *Acanthamoeba* cyst were 0.1% propamidine isethionate (Brolene®) and 0.02% chlorhexidine digluconate.

Heat-killed *E. coli*

*E. coli* colonies were removed from agar surface. The suspension is then pipetted into a universal bottle. *E. coli* suspension is then autoclaved at 121°C for 15 minutes and then stored in the refrigerator at 4°C. *E. coli* suspension serves as a food source for *Acanthamoeba*.  

*Acanthamoeba* cysts suspension

 Mature cyst suspensions were obtained by extending the incubation period up to 14 days. The cysts were gently scraped off using sterile wire loop suspended in 1 ml Page solution, and the number of cysts was counted by Neubauer chamber. The cysts suspension was adjusted to a final concentration of 10⁵ cysts/ml.

Antimicrobial dilutions

Doubling dilutions of 0.1% propamidine isethionate (Brolene®) ranged from 1000 μg/ml to 0.4883 μg/ml and 0.02% chlorhexidine digluconate ranged from 200 μg/ml to 0.0977 μg/ml were performed in 96 well microtitre plates. Stock solution of 200 μg/ml chlorhexidine digluconate was prepared first from its original stock of 200 000 μl/ml. Each row of wells on a microtiter plate was filled with 100 μl of Page saline solution except the first and the last well. The antimicrobial agents were added into the first well and further two-fold serial dilutions of the antimicrobial agents was performed until the 12th well. The cysts suspension serves as a food source for *Acanthamoeba*. Observation was done daily until day 14 to confirm the results before the plates were discarded. The antimicrobial agents were considered effective if the cysts could not undergo excystation to the trophozoite stage. Therefore the absence of trophozoites denote effectiveness of the antimicrobial agents and vice versa.

The minimum cysticidal concentration (MCC) for each antimicrobial agent was determined. MCC is the minimum concentration in which there is no excystment after 14 days. Two or more observers were required to confirm these observations.

RESULTS

The effectiveness of therapeutic dose of 0.1% *propamidine isethionate* (Brolene®) and 0.02% *chlorhexidine digluconate* is shown in Table 2. In this study, both chlorhexidine digluconate and propamidine isethionate were effective on both *Acanthamoeba* isolates.

The minimum cysticidal concentration for both antimicrobial agents against HUKM 74 and HS 62 is shown in Table 3. From this study, with the exposure to 0.1% propamidine isethionate, HUKM 74 showed sensitivity at concentration of 250.00 μg/ml for the first and second test with the mean MCC of 250.00 μg/ml. Meanwhile, the MCC values for HS 62 tested with 0.1% propamidine isethionate at the first and second test were 250.00 μg/ml and 500.00 μg/ml respectively, giving the mean MCC value of 375.00 μg/ml.

From the exposure to 0.02% chlorhexidine digluconate, both HUKM 74 and HS 62 showed their sensitivity at concentration of 50.00 μg/ml, giving the mean MCC value of 50.00 μg/ml.

DISCUSSION

*Acanthamoeba* keratitis (AK) is a rare and serious disease in which the parasites invade the cornea of the eyes. If untreated during its initial phase, the infection can be more severe and results in permanent visual impairment or blindness. The treatment of AK is limited due to the high resistance rate of the cyst to many antimicrobial agents. Thus, the antimicrobial agents used must be both amoebicidal and cysticidal to avoid the re-emergence of infection.

In this study, the therapeutic doses of 0.1% *propamidine isethionate* (Brolene®) and 0.02% *chlorhexidine digluconate* were found to be effective in killing both isolates of *Acanthamoeba* cysts. This is in line with our previous studies (2016 and 2018) but conducted using different *Acanthamoeba* clinical isolates. This finding is supported by Sunada et al. (2014) who also found 0.1% *propamidine isethionate* (Brolene®) and 0.02% *chlorhexidine digluconate* effective as agents against the treatment of AK.

The exposure of cyst isolates to 0.02% chlorhexidine digluconate gave an overall MCC mean value of 50.00 μg/ml which was lower than the mean MCC value of 0.1% propamidine isethionate. The mean MCC values of chlorhexidine in studies done by Kilvington et al. (2002) and Perez Sandoja et al. (2003) were 26.7 ± 17.4 μg/ml and 2.38 ± 1 μg/ml, respectively. The mean MCC values obtained in this study were...
slightly higher than previous studies done by other researchers. One of the factors that lead to the difference in the mean MCC values was due to the use of different *Acanthamoeba* isolates. Martin-Navarro, through his study, noted that the level of effectiveness of antimicrobial agents is different for each isolate of *Acanthamoeba*.

The effectiveness of chlorhexidine as an agent in treating *Acanthamoeba* keratitis can be seen through the binding of its positive charged molecule to the mucopolysaccharide plug of the ostiole on amoeba. This results in membrane cell damage, lysis and death. In contrast to *Acanthamoeba*, results of this study are in line with the study conducted by Sunada et al. (2014) whereby the effectiveness of propamidine on 56 *Acanthamoeba* cyst isolates is less than other antimicrobial agents. In contrast to chlorhexidine, the mechanism of action for propamidine starts from the molecular level. Propamidine acts by inhibiting DNA/RNA synthesis, preventing DNA from functioning as a template which affects the function of polymerases involved in the replication and transcription of DNA, or intercalate into the DNA.

Chlorhexidine is classified under the biguanide group and has been used in treating *Acanthamoeba* keratitis (AK). Compared to other antimicrobial groups, biguanide has the lowest minimal amoebicidal and cysticidal concentrations. Chlorhexidine is probably the most widely used biocide in antiseptic products, particularly in handwashing and oral care products apart from disinfectants and preservatives. This is because it has a broad-spectrum efficacy in killing both Gram positive and Gram negative bacteria. Studies reported that the uptake of chlorhexidine by *E. coli* and *S. aureus* was very rapid and depended on the concentration and pH of chlorhexidine.

Propamidine isethionate is an aromatic diamidine that acts on various types of organisms such as pyrogenic cocci, antibiotic resistance staphyllococi and in some Gram-negative bacilli. This antimicrobial agent has been widely used as a treatment for eye infections such as conjunctivitis and AK. However, the single use of propamidine is often associated with toxic keratoplasty.

In light to the variety of experimental approaches and different outcomes, we think that standardization for *Acanthamoeba* therapeutic agents efficacy testing is clearly needed. In our opinion, in vitro susceptibility test on *Acanthamoeba* isolates can be used for determination of MCCs of therapeutic drugs and should be the method of choice for the screening of new anti-*Acanthamoeba* therapeutic agents.

### CONCLUSION

From this study, it can be concluded that the clinical isolates of *Acanthamoeba* cysts are sensitive towards 0.02% chlorhexidine digluco- nate and 0.1% propamidine isethionate and that both antimicrobial agents are recommended for use in the treatment of AK.

### REFERENCES