Sensitivity of Acanthamoeba Cyst to Antimicrobial Agents

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ABSTRACT

Introduction: Acanthamoeba is an ubiquitous free-living protozoa which causes serious ocular problems. Acanthamoeba keratitis is becoming more prevalent amongst contact lens wearers. The disease can cause loss of vision and blindness if not treated properly. The objective of this research is to study the sensitivity of six Acanthamoeba spp. isolates, of which three were from the clinical isolates (HKL 95, HTH 40 and HS 6) and the remaining three from environmental isolates (TTT 9, TL 3 and SMAL 8) to antimicrobial agents.

Methods: The antimicrobial agents chosen for this purpose were polyhexamethylene biguanide (PHMB) and chlorhexidine. Serial dilutions were perfomed for polyhexamethylene biguanide and chlorhexidine. Cyst suspensions from the chosen isolates were exposed to PHMB and chlorhexidine respectively. After 48 hours incubation time at 30°C, each mixture was filtered and filtration membrane was put onto non-nutrient agar laid with Escherichia coli. The agar plates were incubated for three days at 30°C and examined daily until day 14 to detect the presence of Acanthamoeba trophozoites under the inverted microscope. The presence of trophozoites indicated the ineffectiveness of the antimicrobial agents.

Results: Both of the antimicrobial agents tested were found to be effective against Acanthamoeba cysts from all the test strains. Polyhexamethylene biguanide gave a minimum cysticidal concentration (MCC) mean value of 2.848 µg/mL while chlorhexidine showed MCC mean value at a concentration of 3.988 µg/mL.

Conclusion: It can be concluded that the Acanthamoeba cysts were sensitive to polyhexamethylene biguanide and chlorhexidine.

Keywords: Acanthamoeba, chlorhexidine, polyhexamethylene biguanide, sensitivity

INTRODUCTION

Small free-living amoebae belonging to the genera Acanthamoeba occur world-wide and have been isolated from a wide range of environmental niches including water, soil and dust in air. Because of the widespread distribution of Acanthamoeba, human contact with the organism is inevitable and frequent. Acanthamoeba can cause serious eye infections in contact lens wearers. Proper handling of the contact lens is very important as contaminated contact lens may results in Acanthamoeba infection. Non-sterile products and swimming while wearing contact lens are believed to be risk factors for Acanthamoeba keratitis. Acanthamoeba is resistant to disinfectant, temperature variation and dessication. The treatment for Acanthamoeba keratitis using antimicrobial and antiviral therapy are troublesome. Therefore, this study was done to investigate the effect of certain anti-

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microbial agents against *Acanthamoeba* cyst with the hope that this will benefit everyone especially contact lens wearers, optometrists and ophthalmologists.

**METHODS**

*Acanthamoeba Source*

*Acanthamoeba* isolates were obtained from the *Acanthamoeba* Culture Laboratory, Universiti Kebangsaan Malaysia and the subculture from clinical and environmental isolates.

**Antimicrobial Agents**

The antimicrobial agents tested against *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 expiry dates.

**Sensitivity Test**

The method used was modified from the standard method proposed by Narasimhan *et al.*[5] *Acanthamoeba* cyst suspension was vortexed for about one minute to ensure the cysts were well mixed in the page saline solution. Essentially, double dilutions of each antimicrobial agent were performed in microtitre plates with the five uL cysts at a concentration of $1 \times 10^5$ organisms per $100 \mu l$ of medium per well. Microtitre plates were incubated at 30°C for 48 hours.[5]

A positive control was prepared with page saline solution and another one with cyst mixed with hydrogen peroxide. A negative control on the other hand was prepared with page saline solution only. An antimicrobial agent control was prepared as the negative control as well to ensure they were contamination free. The experiment was processed in duplicate.

The mixture of antimicrobial agent and *Acanthamoeba* cyst, and positive and negative controls were filtered using a filtration unit consisting of millipore, vacuum pump and nitrate cellulose membrane as the main component. A nitrate cellulose membrane measuring 0.45µm was used in this experiment. The microtiter well was rinsed again using page saline solution to detach any remaining cysts. The rinsing was repeated. After filtration, the nitrate cellulose membrane was put onto the non-nutrient agar plates seeded with heat-killed *Escherichia coli* and incubated at 30°C for three days.

Three days later, the membrane was taken out from the agar. 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 results to be negative for the presence of trophozoite. Any trophozoite observed indicates the ineffectiveness of the antimicrobial agents.

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

Polyhexamethylene Biguanide Tested Against Acanthamoeba Cyst

The minimum cysticidal concentration (MCC) values for polyhexamethylene biguanide (PHMB) tested against Acanthamoeba cysts from clinical and environmental isolates are shown in Table 1. The HKL 95 strain gave MCC value of 3.906 µg/mL in the first and second tests, giving a MCC mean value of 3.906 µg/mL. The HTH 49 strain showed a MCC value of 1.953 µg/mL for both first and second tests, giving a MCC mean value of 1.953 µg/mL. The HS 6 strain gave a MCC value of 7.813 µg/mL for the first test and 3.906 µg/mL in the duplicate test. This gave a MCC mean value of 5.859 µg/mL for the particular strain.

On the other hand, TTT 9 strain, an environmental isolate, gave a MCC value of 0.977 µg/mL in the first test and 1.953 µg/mL in the second test, which gave a MCC mean value of 1.465 µg/mL. TL 3 and SMAL 8 showed a MCC value of 1.953 µg/mL for both first and second tests, giving a MCC mean value of 1.953 µg/mL for both strains.

Table 1. Minimum cysticidal concentration (MCC) value for polyhexamethylene biguanide (PHMB) tested against Acanthamoeba isolates

<table>
<thead>
<tr>
<th>Strain</th>
<th>MCC value (µg/mL)</th>
<th>First test</th>
<th>Second test</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>HKL 95</td>
<td>3.906</td>
<td>3.906</td>
<td></td>
<td>3.906</td>
</tr>
<tr>
<td>HTH 49</td>
<td>1.953</td>
<td>1.953</td>
<td></td>
<td>1.953</td>
</tr>
<tr>
<td>HS 6</td>
<td>7.813</td>
<td>3.906</td>
<td></td>
<td>5.859</td>
</tr>
<tr>
<td>TTT 9</td>
<td>0.977</td>
<td>1.953</td>
<td></td>
<td>1.465</td>
</tr>
<tr>
<td>TL 3</td>
<td>1.953</td>
<td>1.953</td>
<td></td>
<td>1.953</td>
</tr>
<tr>
<td>SMAL 8</td>
<td>1.953</td>
<td>1.953</td>
<td></td>
<td>1.953</td>
</tr>
<tr>
<td>MCC Mean</td>
<td></td>
<td></td>
<td></td>
<td>2.848</td>
</tr>
</tbody>
</table>

Chlorhexidine Tested Against Acanthamoeba Cyst

Table 2 shows the minimum cysticidal concentration (MCC) values for chlorhexidine tested against Acanthamoeba cysts from clinical and environmental isolates. The HKL 95 strain gave a MCC value of 3.906 µg/mL in the first and second tests, giving a MCC mean value of 3.906 µg/mL for both tests. The HTH 49 showed a MCC value of 1.953 µg/mL for the first test and 3.906 µg/mL for the second test, giving a MCC mean value of 2.930 µg/mL. The HS 6 strain gave a MCC value of 7.813 µg/mL for the first test and 15.625 µg/mL in the duplicate test. This gave a MCC mean value of 11.719 µg/mL for strain HS 6 when exposed to chlorhexidine.

On the other hand, the environmental isolate, TTT 9, showed a MCC value of 0.977 µg/mL in the first and second tests, giving a MCC mean value of 0.977 µg/mL. TL 3 strain gave a MCC value of 1.953 µg/mL in the first test and 3.906 µg/mL in the second test, with the MCC mean value being 2.930 µg/mL. Finally, strain SMAL 8 showed a MCC value of...
Table 2. Minimum cysticidal concentration (MCC) value for chlorhexidine tested against *Acanthamoeba* isolates

<table>
<thead>
<tr>
<th>Strain</th>
<th>MCC value (µg/mL)</th>
<th>First test</th>
<th>Second test</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>HKL 95</td>
<td>3.906</td>
<td>3.906</td>
<td>3.906</td>
<td></td>
</tr>
<tr>
<td>HTH 49</td>
<td>1.953</td>
<td>3.906</td>
<td>2.930</td>
<td></td>
</tr>
<tr>
<td>HS 6</td>
<td>7.813</td>
<td>15.625</td>
<td>11.719</td>
<td></td>
</tr>
<tr>
<td>TTT 9</td>
<td>0.977</td>
<td>0.977</td>
<td>0.977</td>
<td></td>
</tr>
<tr>
<td>TL 3</td>
<td>1.953</td>
<td>3.906</td>
<td>2.930</td>
<td></td>
</tr>
<tr>
<td>SMAL 8</td>
<td>0.977</td>
<td>1.953</td>
<td>1.465</td>
<td></td>
</tr>
</tbody>
</table>

MCC Mean  3.988

Table 3. Results obtained for positive and negative controls

<table>
<thead>
<tr>
<th>Strain</th>
<th>Control</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30% H_2O_2</td>
<td>Cyst</td>
<td>PAS</td>
</tr>
<tr>
<td>HKL 95</td>
<td>-</td>
<td>+</td>
<td>/</td>
</tr>
<tr>
<td>HTH 49</td>
<td>-</td>
<td>+</td>
<td>/</td>
</tr>
<tr>
<td>HS 6</td>
<td>-</td>
<td>+</td>
<td>/</td>
</tr>
<tr>
<td>TTT 9</td>
<td>-</td>
<td>+</td>
<td>/</td>
</tr>
<tr>
<td>TL 3</td>
<td>-</td>
<td>+</td>
<td>/</td>
</tr>
<tr>
<td>SMAL 8</td>
<td>-</td>
<td>+</td>
<td>/</td>
</tr>
</tbody>
</table>

0.977 µg/mL in the first test and 1.953 µg/mL in the duplicate test, giving a MCC mean value of 1.465 µg/mL.

Positive and Negative Controls

Positive controls were prepared with the cyst exposed to hydrogen peroxide and with Page amebic saline, respectively. As seen in Table 3, the absence of trophozoite was observed until day 14 when the cysts were exposed to hydrogen peroxide. On the other hand, trophozoites were seen in the plate containing cysts added with Page amebic saline.

A negative control was prepared only with Page amebic saline to ensure that the saline was free from any contamination. In this study, the absence of any cyst and trophozoite was observed until day 14 for the negative control. Polyhexamethylene biguanide (PHMB) and chlorhexidine were also tested to ensure they were free from any contamination and in good condition before sensitivity testing was carried out. The absence of any cyst or trophozoite was observed for these plates as seen in Table 4.
Table 4. Results for antimicrobial agent control

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHMB</td>
<td>/</td>
</tr>
<tr>
<td>Chlorhexidine</td>
<td>/</td>
</tr>
</tbody>
</table>

Indicators for Tables 3 and 4:
- Presence of cyst and trophozoite
- Absence of trophozoite
/ Absence of cyst and trophozoite
PAS Page amoebic saline
H₂O₂ Hydrogen peroxide

**DISCUSSION**

Polyhexamethyl biguanide (PHMB) was used initially as the pool disinfectant as it has broad activity over amoeba and bacteria. The anti-amoebic mechanism of PHMB is unknown, but in bacteria, it interrupts the bacteria cell wall causing leakage of the cell wall, followed by cell death. On the other hand, chlorhexidine is classified as a disinfectant or detergent. At a lower concentration, it has been used in the treatment of diseases related to the skin, ear and oral cavity. It has a bacteriostatic and bactericidal activity with microorganism membrane cell destruction and leaves a residual effect which prevents the regrowth of the microorganisms after the application.

Both cationic antiseptics (0.02% PHMB and 0.02% chlorhexidine) interrupt the microorganism membrane cell functions and at a higher concentration, it is found effective in the treatment of *Acanthamoeba* keratitis. Mathers suggests the use of both agents at a concentration of 0.04% and 0.06% respectively which may be successful in patients who did not respond to a lower antimicrobe concentration. A study done by Elder *et al.* in 1994 discovered the MCC values shown by PHMB against *Acanthamoeba* cyst were in the range of 0.49-3.9 µg/mL with the MCC mean value of 2.2 µg/mL. A study by Lee to evaluate the PHMB cysticidal effect on *Acanthamoeba* cyst found the MCC value to be at a concentration of 2.37 µg/mL. In a sensitivity test done by Kilvington, the mean MCC value obtained was 3.2 ± 0.5 µg/mL at a temperature of 32°C.

This study, the data obtained at an incubation temperature of 30°C gave the mean MCC value at 3.906 µg/mL for HKL 95, 1.953 µg/mL for HTH 49, 5.859 µg/mL for HS 6, 1.465 µg/mL for TTT 9, 1.953 µg/mL for TL 3 and SMAL 8. The mean MCC value for all isolates was 2.848 ± 1.71 µg/mL. Although the results differ from that of the other studies, these results do not far exceed or are much lower than the results obtained by other researchers.

Chlorhexidine has also been reported to be effective in the treatment of *Acanthamoeba* keratitis, although there are a few reports which state the existence of resistance among the *Acanthamoeba* isolates tested. As in the case of PHMB, generally chlorhexidine also shows good cysticidal activity. In their study, Elder and colleagues found the MCC value of chlorhexidine at a temperature of 32°C to be 2.77 µg/mL, with a range of 0.49-15.6 µg/mL.
In his study, Lee obtained 7.02 µg/mL as the MCC value of chlorhexidine against *Acanthamoeba* cyst. The mean MCC value in a study done by Kilvington et al. was 26.7 ± 17.4 µg/mL while that of Perez Santoja was 2.38 ± 1 µg/mL. The results obtained in this study was 3.988 µg/mL, and differs from the MCC mean value obtained by other studies. This is maybe due to different isolates used in the study which may show different effectiveness towards chlorhexidine, as well as PHMB.

Although an *in vitro* study found that PHMB and chlorhexidine were effective against *Acanthamoeba* cyst, a relapse may occur with positive *Acanthamoeba* culture in 10% of patients.

*Limitations of the Study*

In this study, the sensitivity test was done only twice as the test was both time and cost-consuming as it needs proper observation of each plate daily until day 14. The *Acanthamoeba* strains tested in this study also needed to be subcultured every 14 days or earlier to ensure *Acanthamoeba* survival. Shortage of food sources and extreme plate dessication kills trophozoites and cysts. So, more agar plates were needed, increasing the research cost indirectly.

**CONCLUSION**

From this study, it can be concluded that the *Acanthamoeba* cysts were sensitive to polyhexamethylene biguanide (PHMB) and chlorhexidine at a minimum cysticidal concentration of 2.848 µg/mL and 3.988 µg/mL respectively. Further investigations can be carried out to test the sensitivity of *Acanthamoeba* from different strains towards different antimicrobial agents and/or eyedrop antibiotics such as myristamidopropyl dimethylamine.

**REFERENCES**


