

## The Effectiveness of Gentamicin against *Acanthamoeba* Cysts in Vitro

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### ABSTRACT

*Acanthamoeba* is a free-living protozoa which causes serious ocular problem. *Acanthamoeba* keratitis is becoming more prevalent amongst contact lens wearers and it can cause loss of vision and blindness if not treated properly. The objective of this research is to determine the effectiveness of gentamicin against six *Acanthamoeba* spp. isolates, of which three were clinical isolates (HS 6, HKL 95, HTH 73) and three environmental isolates (SMAL 7, SMAL 8, TTT 9). Cyst suspension from the chosen isolates were exposed to gentamicin. After 48 hours of incubation at temperature of 30°C and 37°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 37°C and the plates were examined daily until day 14 to look for the presence of *Acanthamoeba* trophozoites under inverted microscope. The presence of trophozoites indicated the ineffectiveness of gentamicin. Gentamicin was found to be effective against *Acanthamoeba* cysts from all the test strains at both incubation temperatures. The minimum cysticidal concentration (MCC) mean value of gentamicin was 0.193 mg/mL at 30°C and 0.229 mg/mL at 37°C. So, we concluded that gentamicin has cysticidal potential towards *Acanthamoeba*.

**Keywords :** *Acanthamoeba*, sensitivity, gentamicin

### INTRODUCTION

Small free-living amoebae belonging to the genera *Acanthamoeba* occurs world-wide. It has been isolated from seawater, ocean sediment and chlorinated swimming pools which can contain up to 10<sup>4</sup> amoebae per litre as *Acanthamoeba* are resistant to chlorine, other types of water and sewage outfalls.<sup>[1]</sup> In addition, a relationship between the presence of *Acanthamoeba* and faecal indicator bacteria has been found in samples of ocean sediment.<sup>[2]</sup> Because of the wide spread distribution of *Acanthamoeba*, human contact with the organism is inevitable and frequent.<sup>[3]</sup>

*Acanthamoeba* may cause a serious eye infection known as *Acanthamoeba* keratitis in healthy individuals which occurs in two separate forms. In the first form, the pathogen is restricted to the epithelium and there is a good chance of recovery. In the second form, the parasite enters the stroma, where it causes necrosis and intense inflammation which leads to blindness if not properly treated.<sup>[4]</sup>

The first-line treatment for *Acanthamoeba* keratitis is topical therapy with biguanides such as polyhexamethylene biguanide or in combination with diamidenes such as propamidine, neomycin (aminoglycosides) and imidazoles. Evaluation of the safety and efficacy of neomycin-polymyxin B-gramicidin ophthalmic solution (Neotracin; Cilag) when it was administered with 0.1% propamidine isethionate has also been carried out.<sup>[5]</sup> However, these drops are not always available and chronic use is associated with significant ocular surface toxicity.<sup>[6]</sup> Furthermore, a combination of drug therapy has been shown to have high success rate only if the disease is diagnosed early. In addition, the treatment for *Acanthamoeba* keratitis using antimicrobial and antiviral therapy is troublesome.<sup>[7]</sup>

Neomycin in combination with other antimicrobial agents is the first line treatment for *Acanthamoeba* keratitis, therefore, this study was performed to investigate the potential efficacies of gentamicin, also an aminoglycosides against *Acanthamoeba* cysts and to determine the effectiveness of gentamicin towards *Acanthamoeba* cysts at different incubation temperature. In this study, the focus is on the cyst of *Acanthamoeba* as it is resistant towards disinfectant, temperature variation and dessication.<sup>[8]</sup> Hopefully this study will help everyone especially contact lens wearers, optometrists and ophthalmologists, so that *Acanthamoeba* keratitis can be avoided or properly treated. Misdiagnosis and mistreatment of *Acanthamoeba* keratitis may lead to resistance development in *Acanthamoeba* cysts and severe eye condition.

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## MATERIALS AND METHODS

### *Acanthamoeba* source

*Acanthamoeba* isolates were obtained from the *Acanthamoeba* Culture Laboratory, Universiti Kebangsaan Malaysia by subculture from clinical (HS 6, HKL 95, HTH 73) and environmental isolates (SMAL 7, SMAL 8, TTT 9).

### Gentamicin

Gentamicin was obtained from The Tun Hussein Onn National Eye Hospital and used before the expiry dates.

### Sensitivity test

The method was modified from the standard method proposed by Narasimhan *et al.* [9]. *Acanthamoeba* cysts suspension was vortexed for one minute to ensure the cysts were thoroughly mixed in the Page Saline solution. Essentially, double dilutions of gentamicin were performed in microtitre plates with five  $\mu\text{L}$  cysts at a concentration of  $1 \times 10^5$  cysts per 100  $\mu\text{L}$  of medium per well. The gentamicin concentration ranges from 0.031 mg/mL to 1 mg/mL. Microtitre plates were then incubated at 30°C and 37°C for 48 hours.

Two positive controls were prepared; one with Page Saline solution mixed with cyst and the other one with hydrogen peroxide mixed with cyst. Two negative controls were also prepared; one with Page Saline solution only and the other one with gentamicin only to ensure they are free from any contaminations. The tests were processed in duplicate.

The mixture of gentamicin and *Acanthamoeba* cyst, positive and negative controls were filtered respectively using the filtration unit which consists of millipore, vacuum pump and nitrate cellulose membrane as the main component. The nitrate cellulose membrane measured 0.45  $\mu\text{m}$ . Microtiter well was rinsed again using Page Saline solution to detach any remaining cyst. Rinsing was done twice. After filtration, the nitrate cellulose membrane was put onto the non-nutrient agar plates seeded with heat-killed *Escherichia coli* as source of food for *Acanthamoeba*. The agar plates were then incubated at 30°C and 37°C for three days.

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 result to be negative for the presence of trophozoite. Any trophozoite observation indicates the ineffectiveness of gentamicin.

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

The minimum cysticidal concentration (MCC) value for gentamicin against *Acanthamoeba* cysts are shown in Table 1. The mean MCC value for all clinical isolates when incubated at 30°C was 0.250 mg/mL. At 37°C, the mean MCC value for isolates HS 6 and HKL 95 was 0.250 mg/mL while for HTH 73 strain, the MCC was 0.375 mg/mL.

**Table 1.** Minimum cysticidal concentration (MCC) value for gentamicin tested against *Acanthamoeba* isolates.

Strain	MCC value (mg/mL)					
	Incubation temperature 30 C			Incubation temperature 37 C		
	First test	Second test	Mean	First test	Second test	Mean
HS 6	0.250	0.25	0.250	0.250	0.250	0.250
HKL 95	0.250	0.250	0.25	0.250	0.250	0.25
HTH 73	0.250	0.250	0.250	0.250	0.5	0.375
SMAL 7	0.125	0.125	0.125	0.250	0.125	0.188
SMAL 8	0.250	0.125	0.188	0.125	0.125	0.125
TTT 9	0.125	0.063	0.094	0.125	0.25	0.188
Mean MCC (mg/mL)			0.193			0.229

The mean MCC for environmental isolates were a bit different from the MCC obtained when gentamicin were tested against the clinical isolates. At incubation temperature of 30°C, the MCC obtained for SMAL 7 strain was 0.125

mg/mL while SMAL 8 was 0.188 mg/mL. TTT 9 strain showed the lowest MCC, which was 0.094 mg/mL. When exposed to gentamicin at 37°C, MCC value obtained for isolate SMAL 7 and TTT 9 was 0.188 mg/mL whilst SMAL 8 was 0.125 mg/mL. Generally, by reading the MCC value, the environmental isolates were found to be more sensitive towards gentamicin compared to the clinical isolates.

Using unpaired t-test, it was found that there was no significant difference in the effectiveness of gentamicin against *Acanthamoeba* cysts at 30 C and 37 C, where  $t(10) = -0.810$ ,  $p > 0.05$ .

#### *Positive and negative controls*

No 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 amoebic saline.

Negative control was prepared with Page amoebic saline alone, to ensure that the saline was free from any contamination. There were no cysts or trophozoites observed until day 14 for the negative control. Gentamicin was also tested to ensure they are free from any contamination and in good condition before sensitivity testing was carried out. No cysts or trophozoites were observed for these plates.

### DISCUSSION

Gentamicin was isolated in 1963 by Weinstein and his colleague from a fungus found in soil, *Micromonospora purpura* (Actinomycetes). It was then introduced in United States in 1969.<sup>[10]</sup>

Gentamicin 40mg/mL contains 40mg gentamicin sulphate and 4.4µg/mL benzalkonium choride as preservative. The mode of action is similar to neomycin as both are aminoglycosides. The aminoglycosides causes codon misread by binding to the ribosomal subunit 30S, stops the peptidyl-tRNA translocation from the recipient to donor area and interrupts protein synthesis.<sup>[11]</sup>

Gentamicin is widely used for the treatment of severe infection caused by bacteria. It is active against gram negative bacteria and *Streptococcus aureus*. It is inactive against anaerobic bacteria and less active against *Streptococcus haemolyticus* and *pneumococcus*.<sup>[12]</sup>

In this study, gentamicin has been serially diluted and tested against *Acanthamoeba* cysts to determine the minimum cysticidal concentration (MCC). Double dilution was performed and sensitivity test of gentamicin against *Acanthamoeba* cysts at 30 C gave mean MCC value of  $0.193 \pm 0.07$  mg/mL for all tested strains. Meanwhile, the mean MCC value obtained from the sensitivity test at 37°C was  $0.229 \pm 0.09$  mg/mL. Ghani<sup>[13]</sup> evaluated the effectiveness of gentamicin on *Acanthamoeba* clinical isolates and discovered the mean MCC as 13.33 mg/ml (range 10-20 mg/ml). The result obtained in our study differed from the mean MCC value obtained by Ghani *et al.* This may be due to different isolates used which may show different levels of sensitivity towards gentamicin.

As there are no other in vitro studies done by other researchers to evaluate the effectiveness of gentamicin against *Acanthamoeba*, so, no detail comparisons can be made with this study. Gentamicin as compared to neomycin has never been used for the treatment of *Acanthamoeba* keratitis. However, in this study, gentamicin was found to have cysticidal potential.

### CONCLUSION

From this study, it can be concluded that gentamicin has potential cysticidal efficacy with minimum cysticidal concentration (MCC) of  $0.193 \pm 0.07$  mg/mL at 30 C and  $0.229 \pm 0.09$  mg/mL at 37°C. Further investigations need to be carried out to evaluate the effectiveness of gentamicin against many other isolates from different sources.

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