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ORIGINAL ARTICLE

Characterizing clinical isolates of *Acanthamoeba castellanii* with high resistance to polyhexamethylene biguanide in Taiwan

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KEYWORDS
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Abstract *Background/Purpose: Acanthamoeba* keratitis (AK), a painful infectious corneal disease, is caused by the free-living pathogenic species *Acanthamoeba*. The symptoms include corneal infiltrate, epithelial, and stromal destruction, and loss of vision. Current treatment generally involves an hourly application of polyhexamethylene biguanide (PHMB) over a period of several days; however, even this is not entirely effective against all strains/isolates. The aims of this study were to confirm the existence of pathogenic strains in Taiwan which are highly resistant to drugs and to characterize the behavior of these strains.
Methods: An *in vitro Acanthamoeba* species culture platform was established to observe the effectiveness of treatment and chart the morphological changes that occur under the effects of drugs using a light microscope and time-lapse recording. Changes in gene expression were examined using reverse transcription polymerase chain reaction (RT-PCR) and real-time PCR.
Results: Over 90% of the standard strain cells (ATCC 30010) were lysed after being treated with PHMB for 1 hour; however, clinical isolates of *Acanthamoeba castellanii* that differed in their susceptibility to the treatment drug were only partly lysed. Following treatment with PHMB, National Cheng Kung University Hospital isolation B (NCKH_B) transformed into a pseudocyst under the effects of drug stress; however, National Cheng Kung University Hospital isolation D (NCKH_D), an isolate with higher tolerance for PHMB, did not transform.

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Conclusion: Our results confirm the existence of clinical isolates of *A. castellanii* with high resistance to PHMB in Taiwan and present the alternative drug tolerance of *A. castellanii* in addition to the transformation of pseudocyst/cyst.

Introduction

Acanthamoeba keratitis (AK) is a serious and very painful, corneal infection caused by pathogenic protozoans of the genus *Acanthamoeba*.[1,2] *Acanthamoeba* species are ubiquitous, free-living protozoa which exist as motile trophozoites and dormant cysts in the wide range of amoebae found in swimming pools, hot tubs, lakes, soil, dust, and drinking fountains.[3] Clinical cases of AK are largely restricted to contact lens wearers who have experienced trauma to their corneal epithelium.[4–7] In the trophozoite stage, *Acanthamoeba* feed on organic particles and microbes, and divide mitotically under optimal conditions. Under exposure to a harsh environment or during periods of stress, the cells can transform into double-walled cysts. The inner wall is comprised of cellulose, and the outer wall consists of proteins and polysaccharides. The largest increases in drug resistance occur during encystation.

The past two decades have seen a remarkable increase in the number of cases of severe ocular inflammation and visual loss caused by *Acanthamoeba* infections. The greater prevalence of *Acanthamoeba* infections has been linked to an increase in contact lens use.[3,8–10] The first two cases of AK in Taiwan were confirmed by corneal biopsy in 1988.[11] According to medical records at National Taiwan University Hospital, Taipei, Taiwan for the period from January 1992 to December 2001, 4.4% of microbial keratitis cases were caused by *Acanthamoeba*.[12] Difficulties in obtaining an early diagnosis and poor response to most antibiotics have led to the use of therapeutic penetrating keratoplasty as a means of eradicating the pathogens in cases of uncontrolled disease. A failure to diagnose this infection early and initiate aggressive treatment can lead to extensive ocular damage, including ring-like corneal infiltration, epithelial destruction, and severe ocular pain. In such cases, enucleation may be required.[9] Confirmation of AK by laboratory diagnosis is the first step in treating this vision-threatening disease, and culturing on non-nutrient agar with an *Escherichia coli* overlay is considered the most effective method of diagnosis. Indeed, rapid diagnosis of pathogens and prompt initiation of appropriate antibiotic therapy are crucial in reducing the morbidity associated with AK.[13]

Currently, the treatment regimen in Taiwan involves hourly drug applications for prolonged periods.[2,14,15] Nonetheless, clinical outcomes tend to be poor, particularly in cases involving drug resistant/tolerant strains. Among drugs used to treat AK, polyhexamethylene biguanide (PHMB) is a membrane-active agent with broad-spectrum antimicrobial efficacy that is commonly used as an antiseptic to deal with keratitis[13–16] in areas such as swimming pools and cooling systems.[17] PHMB is a mixture of polymeric biguanides with an approximate molecular weight range of 400–8000. The structure of PHMB is as follows: $[\text{-}(CH_2)6.NH.C(=NH).NH.C(=NH).NH\text{-}]n$, where $n = 2–40$. In previous studies on prokaryotic cells, PHMB was shown to interact with cytoplasmic membranes to cause the lethal action, nonspecific alterations in membrane integrity, loss of Lipopolysaccharides (LPS), and loss of function in membrane proteins.[10,18,19] PHMB has also been shown to interfere with prokaryotic cell functions by binding to nucleotides,[8] and the drug is further believed to cause the production of large quantities of phosphorus, which accumulates in the nuclei of *Acanthamoeba castellanii* cells as a result of reduced permeability and protein coagulation or the aggregation of phospholipids.[20] Trophozoites have been reported to adsorb more biguanides than do cysts. Complex PHMB-nucleotide formations have been observed near the cell wall and nuclei following drug uptake by *Acanthamoeba*. However, the mechanism underlying the resistance to PHMB by *Acanthamoeba* cells has yet to be elucidated.

It is important to understand the actions of drugs and mechanisms underlying tolerance in *Acanthamoeba* in order to avoid medication failure and improve the effectiveness of therapy for patients suffering from AK. Our results show that *A. castellanii* clinical isolates National Cheng Kung University Hospital isolation B (NCKH_B) and National Cheng Kung University Hospital isolation D (NCKH_D) have higher tolerance toward PHMB than does the standard strain, ATCC 30010. These two isolates differ in their response to PHMB stress: NCKH_B transforms into a pseudocyst to escape the drug, but NCKH_D does not. This study sought to characterize the mechanisms underlying the resistance of *Acanthamoeba* to PHMB in order to facilitate the development of a more effective treatment regimen for AK.

Methods

Culture of *Acanthamoeba* protozoa

Acanthamoeba species were axenically cultured in a proteose peptone-yeast extract-glucose medium, pH 6.5, at 28°C in cell culture flasks. Trophozoites were harvested at the logarithmic growth phase after being cultivated for 3–5 days. The clinical isolates were isolated from the corneal ulcer of patients who were diagnosed as having AK in the National Cheng Kung University Hospital (NCKUH), Tainan, Taiwan.[21] The use of material from patients was reviewed and approved by the Committee of the Institutional Review Board of NCKUH.

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Table 1 Characterization of *Acanthamoeba* clinical isolates.

	Strain	Genotype (18S ribosomal DNA)	Risk factor	Early/late keratitis at presentation	
NCKH_B	*Acanthamoeba castellanii*	T4	SCL	Early	Medical success
NCKH_D	*A. castellanii*	T4	SCL	Late	Surgery

SCL = soft contact lens.

Drug treatment

PHMB was prepared in different concentrations in Page's amoeba saline. To further clarify the appropriate amount of cells, drug concentration, and duration of drug experiment, the counted cells were added in the drug solution and placed on a 100 rpm rotary shaker.

Growth curve and survival rate

Detached cells were centrifuged (1000 *g*) and the pellet was suspended in a 0.5% trypan blue solution in sterile Phosphate-buffered saline (PBS) buffer for 1 minute. Cells were then counted in a Neubauer hemocytometer chamber, and the stained cells were considered dead. Cell counting was performed under a light microscope to determine the numbers of live and dead cells, which were differentiated with the trypan blue dye exclusion viability method. The number of stained cells was subtracted from the total, indicating the death ratio in each interaction condition and time.

Light and fluorescence microscopy

Microscope of Cell (Olympus America, Inc., Melville, NY, USA) was used for the light and fluorescence microscopy observations in this study. Cell is a fully motorized epi-fluorescence microscope equipped with a fast excitation and emission filter wheel allowing for fast multicolor three-dimensional imaging. Image analysis software was used with the microscope.

Total RNA isolation

The Total RNA Extraction Miniprep System (GR1001, Viogene) was used. The cells were centrifuged at 13,500 rpm for 5 minutes, 350 μL RX buffer was then added, the lysate was centrifuged for 5 minutes to spin down insoluble materials, and only the supernatant was used in the following steps. The final volume of the supernatant was determined. An equal volume of 70% ethanol was added to the clear lysate and mixed by vortexing. A Total RNA Mini Column was placed onto a collection tube. Then 700 μL of the ethanol-added sample (including any precipitate) was added into the column and centrifuged for 30—60 seconds. The flow-through was discared. Then the column was washed once with 0.5 mL WF buffer by centrifuging for 30—60 seconds. The flow-through was discared. The column was washed once with 0.7 mL WS buffer by centrifuging for 30—60 seconds. The flow-through was discarded. The column was then centrifuged for another 3 minutes to remove ethanol residue. Next, the column was placed onto a 1.5 mL RNase-free elution tube and 30—50 μL RNase-free ddH_2O was added onto the membrane. The column was allowed to stand for 1 minute, then centrifuged for 1—2 minutes to elute total RNA. The RNA was stored at −70°C. The entire concentration and A260/A280 ratio of mRNA was measured with ND-1000 (NanoDrop).

cDNA synthesis

High capacity cDNA reverse transcription (RT) kits (4368813, Applied Biosystems) were used in this study. To prepare the 2 × RT master mix (per 20 μL reaction), the kit components were allowed to thaw on ice. The volume of components needed to prepare the required number of reactions were calculated: 10 × RT buffer 2.0 μL, 25 × deoxynucleotide (dNTP) mix (100mM) 0.8 μL, 10 × RT random primers 2.0 μL, MultiScribe Reverse Transcriptase 1.0 μL, nuclease-free H_2O 4.2 μL, total per reaction 10.0 μL. RT conditions were set at the following times and temperatures: 25°C 10 minutes, 37°C 120 minutes, 85°C 5 minutes, 4°C ∞. The reaction volume was set to 20 μL.

RT polymerase chain reaction

One step RT polymerase chain reaction (RT-PCR) was performed with SuperScript One-Step RT-PCR with Platinum Taq kit (12574-018, Invitrogen) to investigate the gene expression of *Acanthamoeba spp.* of the cyst-specific protein (CSP21), cellulose synthase II (CSII).[22] All of the cDNA was synthesized from 1 μg of total RNA of *Acanthamoeba*. RT-PCR products were separated on Ethidium bromide (EtBR)-stained gel after electrophoresis in 1.0—1.2% agarose. The primers sequences for CSII were ACGAGATGGAGAGCATCCAG (forward) and CAGCACCTCGGTGTTGTAGA (reverse), and for CSP21 ACTCCTTCAGGTCGTCCA (forward) and TTCTCCTCGAGGCCCATGTC (reverse).

Results

A. castellanii was collected from the corneal ulcers of patients diagnosed with AK at NCKUH. The collected *A. castellanii* presented a variety of pathogenicities and various degrees of susceptibility to PHMB. After the culture, 18s ribosomal DNA sequences (Rns) were used for sequencing and genotyping. Identification of *Acanthamoeba* Rns genotypes was based on sequences of 113 bp within the genus-specific amplicon ASA.S1. From the results, we confirmed two clinical isolates: NCKH_B, which is susceptible to PHMB treatment, and NCKH_D, which is not susceptible to PHMB

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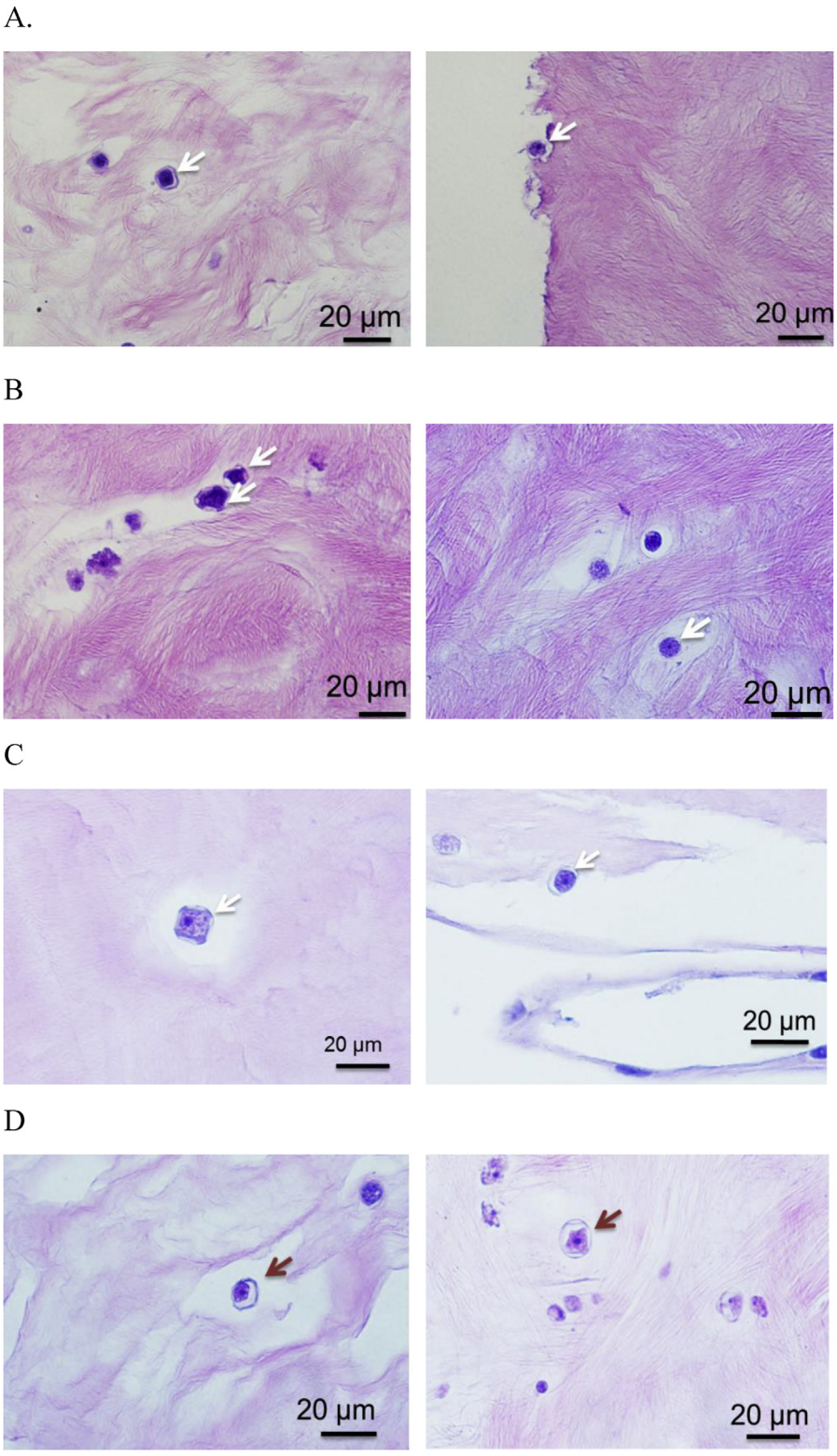

Figure 1. Human cornea tissue infected by National Cheng Kung University Hospital isolation B (NCKH_B): (A) 48 hours postinfection; (B) 96 hours postinfection and NCKH_D (C) 48 hours postinfection; (D) 96 hours postinfection. The arrows indicate the position of the *Acanthamoeba* cell.

treatment. The characterization of these two clinical isolates is listed in Table 1. Both of these isolates belong to Rns genotype T4 strains, the predominant Rns genotype found in AK infections (data not shown). To evaluate the ability of these isolates to spread infection, we cocultured the *A. castellanii* with human cornea tissue using the ATCC 30010 strain (standard nonpathogenic strain) as a control. Staining with hematoxylin and eosin was performed on infected

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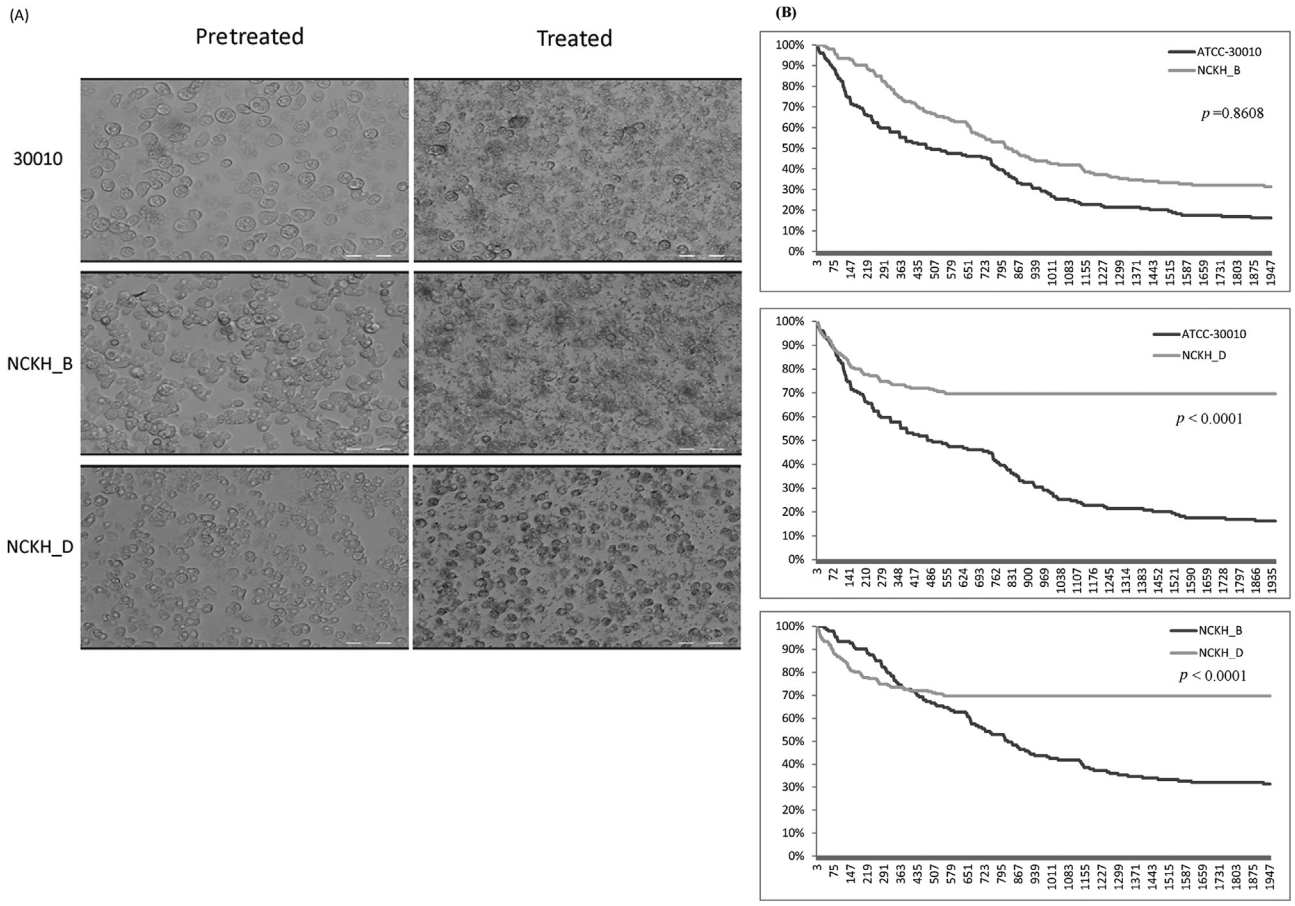

Figure 2. The light observation for effect imaging of addition of 0.01% polyhexamethylene biguanide (PHMB) to the live *Acanthamoeba* strains by time-lapse recording: (A) the captured images; (B) the statistical results of the survival rate (log-rank test on Kaplan—Meier estimates of survival).

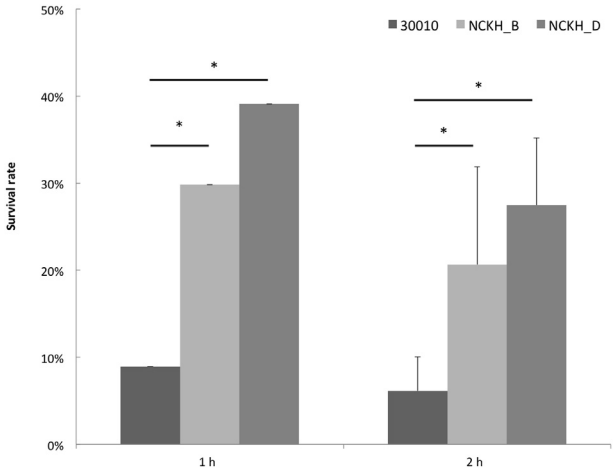

Figure 3. Effects of *Acanthamoeba* strains during the progress of polyhexamethylene biguanide (PHMB) treatment. * Means are significantly different at $p < 0.05$ by Student *t* test.

tissues. Results of our analysis revealed that *A. castellanii* cells were present in all tissue sections infected with NCKH_B for 48 hours (Figure 1A) or 96 hours (Figure 1B) and in tissue sections infected with NCKH_D for 48 hours (Figure 1C) or 96 hours (Figure 1D). In the tissues cocultured with the ATCC 30010 control, no *A. castellanii* cells were observed.

To elucidate the effectiveness of PHMB treatment, we established an *in vitro* drug test platform for species of *Acanthamoeba*. This treatment involved the hourly application of 0.02% PHMB in the form of eye drops (200 μg/mL) over a prolonged period. This experiment was conducted in accordance with a previous report and counted the reliable PHMB concentration in the eyes. Because there are tears in eyes, the initial PHMB concentration in the eye must be < 200 μg/mL and decreased rapidly (the turnover rate is 8.2% per minute).[23] Seven minutes after applying the eye drops, the drug concentration is expected to drop below 100 μg/mL. To mimic clinical treatment situations, the *A. castellanii* isolates were treated with a similar concentration of PHMB as that encountered after administration and serial dilution in an actual eye. Our results indicated that at 100 μg/mL, the effectiveness of PHMB in the treatment of *A. castellanii* was close to that observed when PHMB is administered to patients using an eye-dropper. To identify the susceptibilities of ATCC30010, NCKH_B, and NCKH_D to PHMB, we treated parasites with 100 μg/mL PHMB and monitored the results using time-lapse recording obtained with a differential interference contrast microscope.

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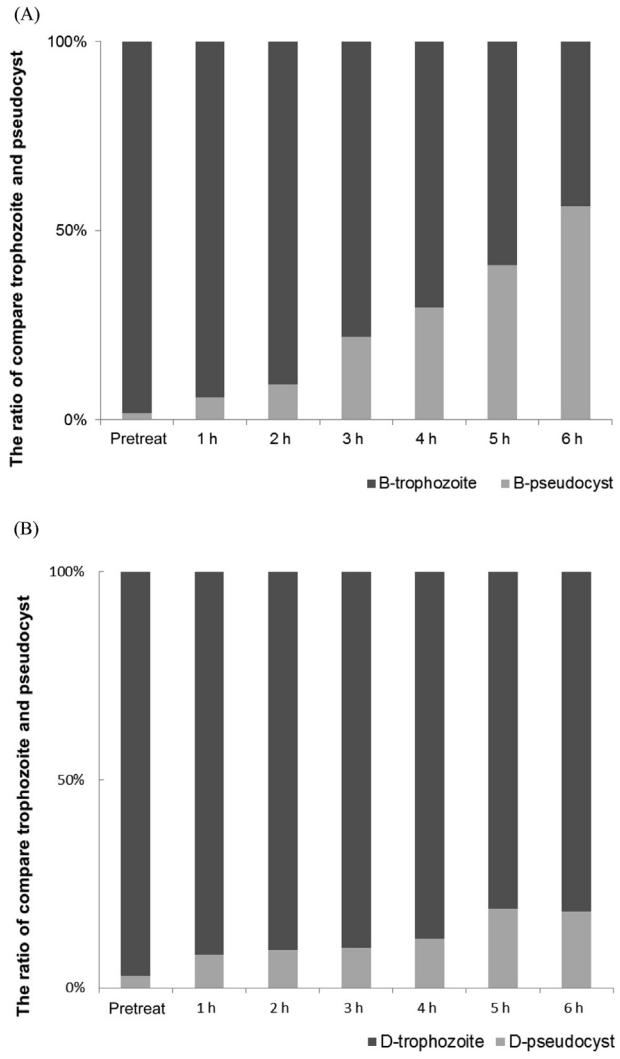

Figure 4. The correlative ratio of pseudocysts-trophozoites to (A) NCKH_B and (B) NCKH_D during the progress of polyhexamethylene biguanide (PHMB) treatment.

Images were taken every 3 seconds over a period of 32.5 minutes. To quantify the number of live cells in the images and calculate the survival rate of cells (as per description in Methods) during the experiments could reveal the effect of the drug on the *Acanthamoeba* strains/isolates. From the survival analysis results by the Kaplan—Meier method and log-rank test,[24] the tolerance of both clinical isolates to PHMB exceeded that of ATCC30010, with NCKH_D presenting the greatest tolerance (Figure 2).

In order to characterize the mode(s) of live *A. castellanii* cells in resisting the stress associated with PHMB treatment, we extended the treatment period and monitored the survival rate every hour. After being treated with PHMB at a concentration of 100 μg/mL, > 90% of the cells belonging to the standard strain ATCC 30010 had been lysed. By contrast, only 70% of the NCKH_B and 60% of the NCKH_D had been lysed within the first hour. After 2 hours, about 80% of NCKH_B isolates and 70% of NCKH_D isolates were lysed (Figure 3). Between 3 hours and 6 hours, survival rates did not change significantly from those observed after

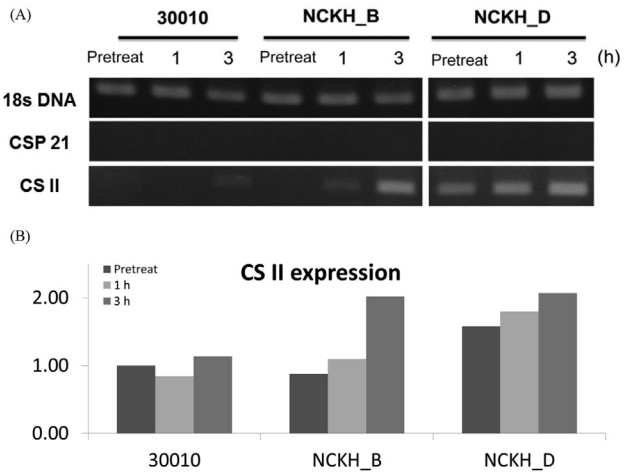

Figure 5. Reverse transcription polymerase chain reaction (RT-PCR) analysis of the gene expression level of polyhexamethylene biguanide (PHMB)-treated clinical isolates NCKH_B, NCKH_D, and ATCC 30010. Csp21, the marker of cyst; CS II (cellulose synthase II), the marker of pseudocyst; the 18S rDNA expression, used as a control.

the 2nd hour (data not shown). Throughout the course of PHMB treatment, a population of the NCKH_B and the NCKH_D isolates were found to survive, having transformed into pseudocysts. This behavior was not observed in the ATCC strain. Throughout the PHMB treatment period, the correlative ratio of pseudocysts-trophozoites to live NCKH_B cells increased; however, this was not observed in the NCKH_D cells (Figure 4). To confirm the different responses of the isolates, we checked the correlative gene expression level of the *A. castellanii* encystation and pseudocyst transformation.[22] Our results show that gene expression for Csp21, the marker associated with cyst transformation, was not detected (Figure 5). During the progress of PHMB treatment, the gene expression levels of CSII, the key enzyme in pseudocyst transformation, were higher in the NCKH_B than in ATCC 30010. Interestingly, the expression of CSII in NCKH_D during the progress of PHMB

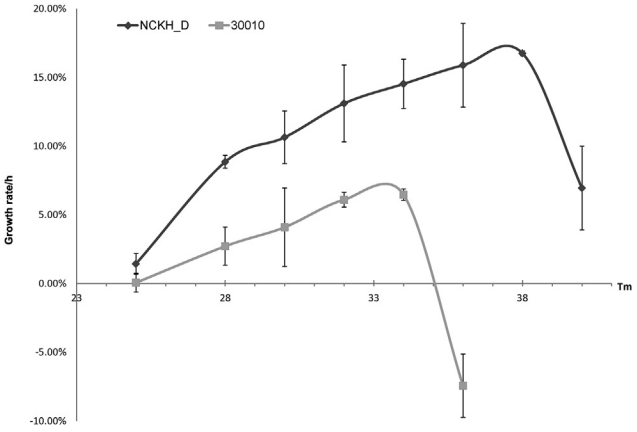

Figure 6. Growth rates (percentage increase per hour) of ATCC 30010 and NCKH_D at different temperatures.

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treatment remained at a high level; however, the ratio of pseudocysts-trophozoites to live NCKH_D cells did not appear to change significantly.

In a previous study, Nielsen et al[25] reported that the optimal growth temperature for the four strains of *A. castellanii* (32°C) is close to the surface temperature of the human cornea. We tested the growth rates of NCKH_D and ATCC 30010 at different temperatures and found that the optimal growth temperature of NCKH_D is around 38°C (Figure 6). It is not only higher than ATCC 30010, but also than the strains which Nielsen et al[25] reported. In addition, Jensen et al[26] reported that doubling time for *A. castellanii* is as low as 6 hours, with a maximal growth rate of 11.5% per hour. From our results, the maximal growth rate of the NCKU_D is 16.76% per hour. The clinical isolate, NCKH_D, is a rapid-growth pathogenic strain with high PHMB resistance.

Discussion

AK is a major cause of severe ocular inflammation, epithelial and stromal destruction, and loss of vision, which can eventually necessitate eyeball removal. Unfortunately, none of the medical or surgical treatments for AK have proven consistently reliable. At present, treatment involves hourly applications of PHMB over a prolonged period; however, even this aggressive approach is unable to ensure anything other than a poor prognosis. Obtaining reliable data related to the efficacy of treatment methods and the isolation of clinical strains with various degrees of drug susceptibility, are the first crucial steps in developing a more effective treatment regimen for AK. In this study, clinical isolates of *A. castellanii* were collected, their susceptibility to PHMB identified, and their behavior when challenged with this drug was characterized.

Previous researchers have reported that PHMB acts on intracystic amoebae by shrinking them and by separating the plasma membrane from the endocystic wall.[27] The images obtained in this study show that PHMB triggers the rupturing of Acanthamoebal trophozoite cells into fragments. Extending PHMB treatment resulted in an increase in the population of surviving NCKH_B cells, which had transformed into pseudocysts. Clearly, the PHMB resistance of NCKH_B far exceeds that of the ATCC30010 strain.

The highest PHMB tolerant isolate, NCKH_D, did not depend on transforming to escape the drug mainly, but the RNA expression levels of CSII were constant in high levels, even without drug treatment. From our observation, most of the live NCKH_D cells were non-motile to maintain shape during the PHMB treating. When we removed the drug and replaced the fresh medium, a part of the NCKH_D cells soon regained activity (data not shown). Clarification of the PHMB tolerance/resistance mechanisms of highly tolerant isolates like NCKH_D is needed.

Clinical isolates of *A. castellanii* obtained in Taiwan, which show a pronounced tolerance for PHMB, were characterized in this study. Furthermore, we present the alternative drug tolerance of *A. castellanii* in addition to the transformation of pseudocysts/cysts. This study has begun to elucidate the behavior of *A. castellanii* in resisting PHMB; our findings are a first step towards improving clinical therapies for patients infected with drug-resistant AK.

Conflicts of interest

All authors declare no conflicts of interest.

Acknowledgments

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References

1. McCulley JP, Alizadeh H, Niederkorn JY. *Acanthamoeba* keratitis. *CLAO J* 1995;**21**:73—6.
2. Visvesvara GS, Moura H, Schuster FL. Pathogenic and opportunistic free-living amoebae: *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri*, and *Sappinia diploidea*. *FEMS Immunol Med Microbiol* 2007;**50**:1—26.
3. Brown TJ, Cursons RT, Keys EA. Amoebae from Antarctic soil and water. *Appl Environ Microbiol* 1982;**44**:491—3.
4. Centers for Disease C. *Acanthamoeba* keratitis in soft-contact-lens wearers. *MMWR Morb Mortal Wkly Rep* 1987;**36**:397—8. 403—4.
5. Morlet N, Duguid G, Radford C, Matheson M, Dart J. Incidence of *Acanthamoeba* keratitis associated with contact lens wear. *Lancet* 1997;**350**:414.
6. Paszko-Kolva C, Yamamoto H, Shahamat M, Sawyer TK, Morris G, Colwell RR. Isolation of amoebae and Pseudomonas and Legionella spp. from eyewash stations. *Appl Environ Microbiol* 1991;**57**:163—7.
7. van Klink F, Alizadeh H, He Y, Mellon JA, Silvany RE, McCulley JP, et al. The role of contact lenses, trauma, and Langerhans cells in a Chinese hamster model of *Acanthamoeba* keratitis. *Invest Ophthalmol Vis Sci* 1993;**34**:1937—44.
8. Allen MJ, Morby AP, White GF. Cooperativity in the binding of the cationic biocide polyhexamethylene biguanide to nucleic acids. *Biochem Biophys Res Commun* 2004;**318**:397—404.
9. Auran JD, Starr MB, Jakobiec FA. *Acanthamoeba* keratitis. A review of the literature. *Cornea* 1987;**6**:2—26.
10. Broxton P, Woodcock PM, Gilbert P. A study of the antibacterial activity of some polyhexamethylene biguanides towards Escherichia coli ATCC 8739. *J Appl Bacteriol* 1983;**54**:345—53.
11. Chan MY, Ca TC. *Acanthamoeba* keratitis: report of the first two cases in Taiwan. *ACTA Societatis Ophthalmologicae Sinicae* 1988;**27**:172—8.
12. Fong CF, Tseng CH, Hu FR, Wang IJ, Chen WL, Hou YC. Clinical characteristics of microbial keratitis in a university hospital in Taiwan. *Am J Ophthalmol* 2004;**137**:329—36.
13. Donoso R, Mura JJ, Lopez M. [*Acanthamoeba* keratitis treated with propamidine and polyhexamethyl biguanide (PHMB)]. *Rev Med Chil* 2002;**130**:396—401.
14. McDonnell G, Russell AD. Antiseptics and disinfectants: activity, action, and resistance. *Clin Microbiol Rev* 1999;**12**:147—79.
15. Gray TB, Gross KA, Cursons RT, Shewan JF. *Acanthamoeba* keratitis: a sobering case and a promising new treatment. *Aust N Z J Ophthalmol* 1994;**22**:73—6.
16. Narasimhan S, Madhavan HN, Lily Therese K. Development and application of an in vitro susceptibility test for *Acanthamoeba* species isolated from keratitis to polyhexamethylene biguanide and chlorhexidine. *Cornea* 2002;**21**:203—5.

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17. Kusnetsov JM, Tulkki AI, Ahonen HE, Martikainen PJ. Efficacy of three prevention strategies against legionella in cooling water systems. *J Appl Microbiol* 1997;**82**:763–8.
18. Broxton P, Woodcock PM, Heatley F, Gilbert P. Interaction of some polyhexamethylene biguanides and membrane phospholipids in Escherichia coli. *J Appl Bacteriol* 1984;**57**:115–24.
19. Gilbert P, Pemberton D, Wilkinson DE. Synergism within polyhexamethylene biguanide biocide formulations. *J Appl Bacteriol* 1990;**69**:593–8.
20. Khunkitti W, Hann AC, Lloyd D, Furr JR, Russell AD. X-ray microanalysis of chlorine and phosphorus content in biguanide-treated *Acanthamoeba* castellanii. *J Appl Microbiol* 1999;**86**:453–9.
21. Li ML, Shih MH, Huang FC, Tseng SH, Chen CC. Treatment of early *Acanthamoeba* keratitis with alcohol-assisted epithelial debridement. *Cornea* 2012;**31**:442–6.
22. Kliescikova J, Kulda J, Nohynkova E. Stress-induced pseudocyst formation–a newly identified mechanism of protection against organic solvents in acanthamoebae of the T4 genotype. *Protist* 2011;**162**:58–69.
23. Norn M. The effects of drugs on tear flow. *Trans Ophthalmol Soc U K* 1985;**104**(Pt 4):410–4.
24. Xie J, Liu C. Adjusted Kaplan-Meier estimator and log-rank test with inverse probability of treatment weighting for survival data. *Stat Med* 2005;**24**:3089–110.
25. Nielsen MK, Nielsen K, Hjortdal J, Sorensen UB. Temperature limitation may explain the containment of the trophozoites in the cornea during *Acanthamoeba castellanii* keratitis. *Parasitol Res* 2014;**113**:4349–53.
26. Jensen T, Barnes WG, Meyers D. Axenic cultivation of large populations of *Acanthamoeba castellanii* (JBM). *J Parasitol* 1970;**56**:904–6.
27. Kong HH, Chung DI. Ultrastructural changes of *Acanthamoeba* cyst of clinical isolates after treatment with minimal cysticidal concentration of polyhexamethylene biguanide. *Korean J Parasitol* 1998;**36**:7–13.

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