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ORIGINAL PAPER

Evaluating the in vitro efficacy of gatifloxacin, levofloxacin and gentamicin against *Acanthamoeba* cysts

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Abstract
Purpose To evaluate the in vitro efficacy of three commercial ophthalmic solutions (gatifloxacin, levofloxacin and gentamicin) against cysts of *Acanthamoeba* species.
Design Experimental study
Methods *Acanthamoeba* cysts belonging to genotypes T3, T4 and T5 were incubated with three ophthalmic solutions for different periods of time; 1, 24, 48 and 72 h at 37 °C. After incubation, treated cysts were stained with trypan blue and counted to express the percent of growth inhibition. Additionally, the viability of treated cysts was assessed by culturing them in PYG medium at 30 °C for 72 h as well as on non-nutrient agar plates at 30 °C for 1 month.
Results *Acanthamoeba* cysts of all genotypes were susceptible to gentamicin and gatifloxacin after exposure for 1 h and 24 h, respectively, and for levofloxacin, cysts of all genotypes were resistant to levofloxacin even after 72 h of incubation. Gentamicin and gatifloxacin showed statistically highly significant difference ($P < 0.001$), and levofloxacin showed statistically significant difference ($P < 0.05$) in comparison to non-treated control.
Conclusions Gentamicin and gatifloxacin were highly effective against *Acanthamoeba* cysts. Although our results should be confirmed in animal models, this result will guide the choice of the appropriate ophthalmic drugs for early treatment of eye infection caused by *Acanthamoeba* spp.

Keywords Gatifloxacin · Gentamicin · Levofloxacin · *Acanthamoeba* cysts · Ophthalmic drugs

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Introduction

Free-living amoebae of the genus *Acanthamoeba* are the worldwide distribution in a variety of habitats such as soil, water and even in the air [1]. There are two forms in their life cycle, a vegetative trophozoite stage and a highly resistant cyst stage. Trophozoite can develop into protective cyst form when environmental conditions become adverse. Based on the variation of nucleotide sequences of the Diagnostic fragment 3 (DF3) region of the 18S rRNA gene, *Acanthamoeba* spp. have been classified into 20 genotypes, T1–T20

[2]. *Acanthamoeba* species can cause serious infections, granulomatous amoebic encephalitis (GAE) which is rare but high fatality rate in immunocompromised persons and amoebic keratitis (AK) in immunocompetent persons, which can result in poor vision or blindness if delay in diagnosis and inadequate treatment. In developing countries, AK has been reported in people previously suffering from corneal ulcers and having contact with contaminated water and soil, even without wearing contact lenses [3]. In Thailand, AK cases have been reported among both contact lens users and non-contact lens users. Twenty-four AK cases occurred in Thailand between 1996 and 2006 [4, 5], of which five were reported in 1999 [6]. Misdiagnosis of AK is common because signs and symptoms are not specific and can mimic bacteria, fungal or viral keratitis [1] together with lacking of consensus of AK diagnosis, these lead to AK remains significant. Therefore, AK treatment depends on clinicians' experience. At present, the first-line drugs for AK are biguanides, such as chlorhexidine and PHMB, and diamidines such as propamidine isethionate and neomycin. Although the treatment of AK often requires a combination of biguanides and diamidines [7], not all AK cases are cured with these [8]. Trophozoites are usually susceptible to biocides including therapeutic drugs. The major problem for AK treatment is therefore, unable to eliminate cyst stage completely [9]. Various therapeutic drugs have been studied in vitro by focusing on cysticidal efficacy, as effectiveness is essential for subsequent studies in vivo therapy of *Acanthamoeba* keratitis [10–12]. However, their protocols have not been standardized for using and no eyedrops preparations are commercially available. Moreover, there are no fully effective against all strains in the available treatments. Therefore, it is necessary to study new therapeutic agents to be the alternative drugs for AK treatment.

The aim of this study was to evaluate the in vitro efficacy of three commercially available ophthalmic solutions (gatifloxacin, levofloxacin and gentamicin) against cysts of three *Acanthamoeba* strains, Thailand isolates. These ophthalmic drugs are usually used to treat keratitis caused by bacteria. Chlorhexidine, first-line AK treatment, was used as a reference drug. This study will guide the choice of drug to treat early stages of eye disease caused by *Acanthamoeba* spp.

Methods

Acanthamoeba strains and cysts preparation

Three environmental strains of *Acanthamoeba* were examined: GenBank accession numbers KT897271 (T3), KT897265 (T4) and KT897268 (T5) [13]. Each strain was cultured onto non-nutrient agar plates which were seeded with 5 µL of heat-killed *Escherichia coli* and incubated at 30 °C for 3 weeks. The cysts were harvested by washing plates with phosphate buffered saline (PBS) and decanted into 15 mL tubes which were centrifuged at 3000 rpm for 5 min. The supernatant was discarded and 0.5% sodium dodecyl sulfate (SDS) added for 10 min to lyse immature cysts and then the tubes were centrifuged at 3000 rpm for 5 min. The SDS was washed out twice using PBS. Finally, cysts were counted with a hemocytometer and standardized to a concentration of 20×10^4 cysts/mL.

Chemicals

Four antimicrobial agents were tested: chlorhexidine (Sigma-Aldrich, USA) was prepared at the commercially available concentrations (0.02%) and used as reference drug, and three ophthalmic solutions, 0.3% gentamicin (3 mg/mL) (Seng Thai Pharmaceutical Laboratory, Bangkok, Thailand), 0.3% gatifloxacin (3 mg/mL) (Allergan Sales, TX, USA) and 0.5% levofloxacin (5 mg/mL) (Santen Pharmaceutical Laboratory, Osaka, Japan).

Evaluation of the cysticidal activity

Qualification assays

Experiments for eyedrops testing on cysts were performed in the sterile microtube. Briefly, 100 µL of each of calibrated *Acanthamoeba* strain (20×10^4 cysts/mL) was incubated with 100 µL of each eyedrop for 1, 24, 48 and 72 h at 37 °C, each in duplicate. After incubation, the suspension was washed twice with PBS to eliminate residual drugs and resuspended with 100 µL PBS. The cyst viability was examined by taking 20 µL of sample into NNA medium coated with heated *E. coli* and incubated at 30 °C, examining the samples every day by microscope for 1 month. The remaining content was then cultured in 500 µL axenically PYG medium

containing 50 µg/mL enrofloxacin (General Drugs House, Bangkok, Thailand) and incubated at 30 °C for 72 h in 96-well microtiter plate and examined by inverted microscope. The presence of trophozoite was considered as drug-resistant strain.

Quantification assays

For growth inhibition evaluation, 100 µL of treated cysts of each eyedrop were stained with 100 µL 0.4% trypan blue for 10 min, unstained cysts were viable and stained cysts were nonviable [14]. Viable and nonviable cysts were then counted using the hemocytometer at 1, 24, 48 and 72 h. The percent of growth inhibition was calculated: Percent of growth inhibition = $a - b/a \times 100$ (a mean number of non-treated cysts, b is the mean number of treated cysts) [15].

All assays, both qualification and quantification were repeated five times, each in duplicate, for each strain. In addition, control containing cysts treated with 0.02% chlorhexidine gluconate as a reference drug control and cysts in PBS as non-treated control were performed to the same procedure. The effect of eyedrops on cysts for morphological observation was examined by a light microscope (× 40).

Data analysis

Data management and analysis were made using SPSS version 19.0 for Windows. Cyst number was calculated as mean ± SD and percent of growth inhibition. The mean numbers were analyzed by using analysis of variance (ANOVA) followed by the Tukey test for post hoc comparisons.

Results

Cysticidal activity assays

The efficacy of three ophthalmic drugs (0.3% gentamicin, 0.3% gatifloxacin and 0.5% levofloxacin) was evaluated for their cysticidal activity against three *Acanthamoeba* strains at different time points (1, 24,

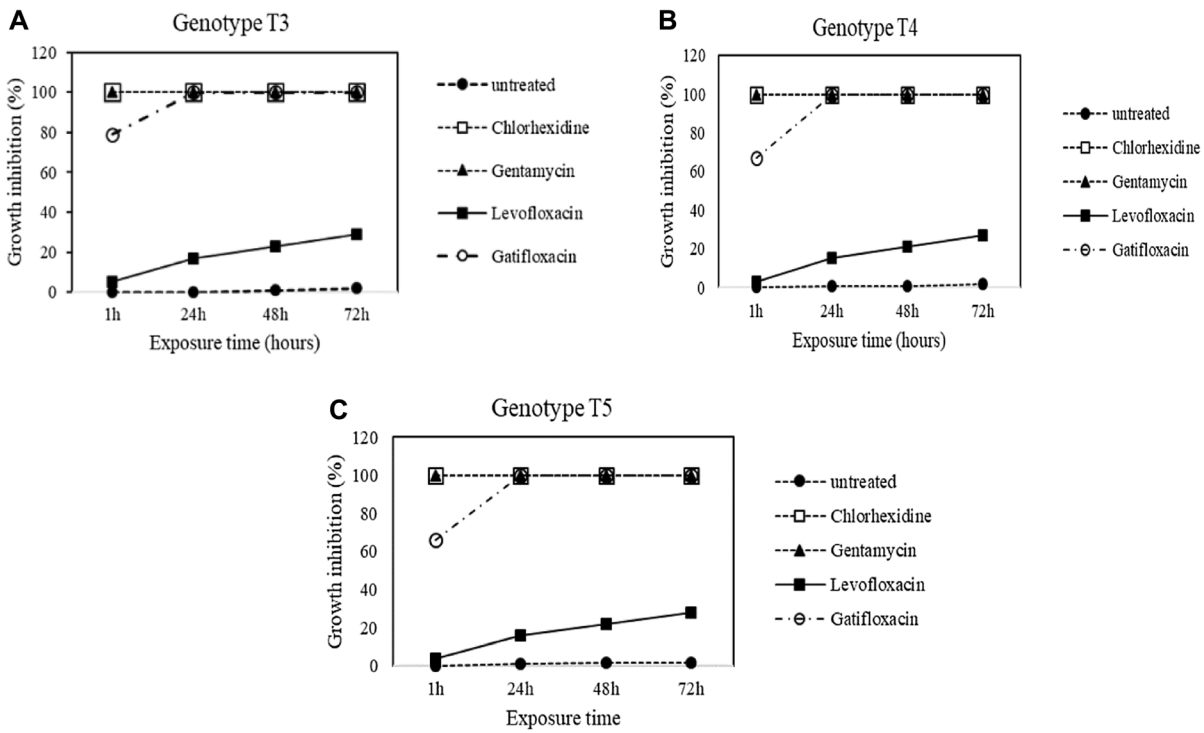

Fig. 1 Percent of growth inhibition of *Acanthamoeba* species after exposure to 0.02% chlorhexidine, 0.3% gentamicin, 0.3% gatifloxacin and 0.5% levofloxacin for different periods of time. **a** *Acanthamoeba* genotype T3, **b** *Acanthamoeba* genotype T4 and **c** *Acanthamoeba* genotype T5

Table 1 Percentage of growth inhibition and (Mean ± SD) of *Acanthamoeba* cyst after exposure to each ophthalmic drug in different incubation times

Acanthamoeba genotype	Incubation time (h)	Mean ± SD and percentage of growth inhibition of *Acanthamoeba* cyst									
		Gatifloxacin		Levofloxacin		Gentamicin		PBS (non-treated control)		Chlorhexidine (positive control)	
		Mean ± SD	%	Mean ± SD	%	Mean ± SD	%	Mean ± SD	%	Mean ± SD	%
T3	1	4.18 ± 0.035**	79.1	19.16 ± 0.012•	4.21	0.00**	100	19.96 ± 0.008	0.2	0.00**	100
	24	0.00**	100	16.60 ± 0.031*	17.0	0.00**	100	19.95 ± 0.016	0.25	0.00**	100
	48	0.00**	100	15.38 ± 0.007*	23.1	0.00**	100	19.80 ± 0.008	1	0.00**	100
	72	0.00**	100	14.20 ± 0.008*	29	0.00**	100	19.60 ± 0.021	2	0.00**	100
T4	1	6.6 ± 0.007**	67	19.40 ± 0.018•	3	0.00**	100	19.94 ± 0.006	0.3	0.00**	100
	24	0.00**	100	16.96 ± 0.025*	15.2	0.00**	100	19.88 ± 0.008	0.6	0.00**	100
	48	0.00**	100	15.80 ± 0.007*	21	0.00**	100	19.76 ± 0.007	1.2	0.00**	100
	72	0.00**	100	14.54 ± 0.016*	27.3	0.00**	100	19.54 ± 0.009	2.3	0.00**	100
T5	1	6.8 ± 0.019**	66	19.20 ± 0.008•	4	0.00**	100	19.94 ± 0.007	0.3	0.00**	100
	24	0.00**	100	16.80 ± 0.007*	16	0.00**	100	19.59 ± 0.005	0.3	0.00**	100
	48	0.00**	100	15.74 ± 0.026*	21.3	0.00**	100	19.64 ± 0.007	1.8	0.00**	100
	72	0.00**	100	14.52 ± 0.035*	27.4	0.00**	100	19.52 ± 0.008	2.4	0.00**	100

SD Standard deviation

**$P < 0.001$ statistically highly significant difference, *$P < 0.05$ statistically significant difference, •$P > 0.05$ no statistically significant difference

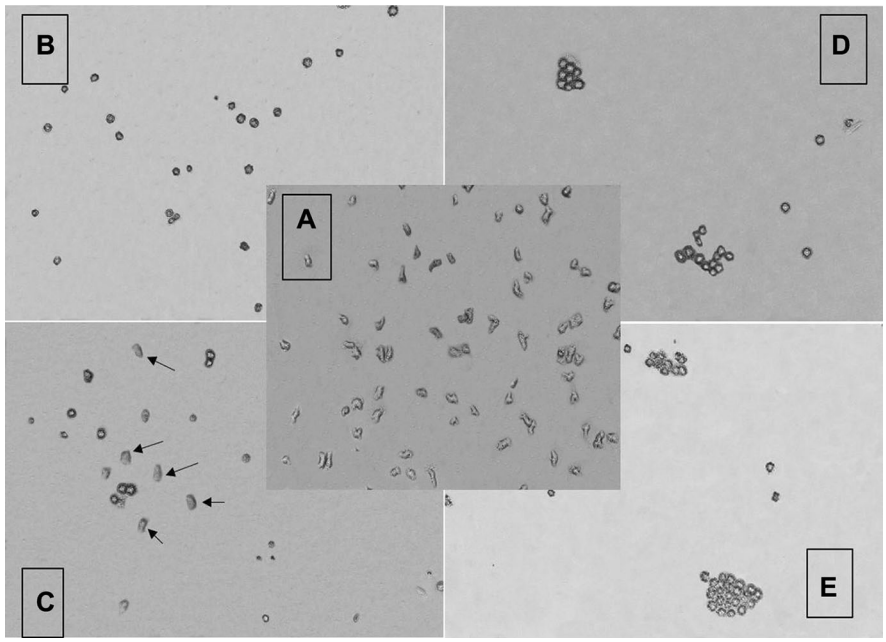

Fig. 2 *Acanthamoeba* cysts of T4 genotype treated with each eyedrop for 72 h, and the viability were tested by PYG culture for 72 h and observed by inverted microscopy (× 20). **a** Non-treated control, **b** 0.02% chlorhexidine, **c** 0.5% levofloxacin, **d** 0.3% gentamicin, **e** 0.3% gatifloxacin

48, 72 h). After incubation, the viability was determined using trypan blue staining and counting for growth inhibition calculation. The results showed that *Acanthamoeba* cysts of all strains were susceptible to gentamicin and gatifloxacin when incubated for at least 1 and 24 h, respectively. Levofloxacin was the least effective of the three drugs tested. No genotypes were completely killed, even after 72 h incubation. The results of growth inhibition are shown in Fig. 1, and mean number of cysts (mean ± SD) after exposure to drugs are shown in Table 1. After statistical analysis, gentamicin and gatifloxacin showed highly statistically significant difference ($P < 0.001$) and levofloxacin showed statistically significant difference ($P < 0.05$) as compared with non-treated control. Gentamicin was the most effective ophthalmic drugs against cysts of all three genotypes of *Acanthamoeba* in our study ($P < 0.001$).

In the present study, treated cysts were inoculated into PYG medium and on NNA medium overlaid with heated *E. coli* for qualification viability tests. The results from NNA culture corresponded to PYG culture; gentamicin and gatifloxacin were highly effective against *Acanthamoeba* cysts of all genotypes after exposure 1 h and 24 h, respectively. In contrast, levofloxacin had the least effectiveness against all three genotypes. Even after incubation with this drug for 72 h, cysts of all three genotypes were capable of yielding trophozoites in the viability test. Figure 2 showed the results of *Acanthamoeba* cysts of genotype T4 treated with each eyedrop for 72 h and then cultured in PYG medium. After observation by inverted microscope (× 20), the results revealed there were no excysted trophozoites in samples treated with 0.02% chlorhexidine, 0.3% gentamicin and 0.3% gatifloxacin, but presented in sample treated with 0.5% levofloxacin. The other two strains, T3 and T5 genotypes, also revealed the same results (data not shown). The altered morphology with cytoplasm destruction after 24 h of incubation with each effective eyedrop, chlorhexidine, gatifloxacin and gentamicin is shown in Fig. 3.

Discussion

Our study has presented the susceptibility of cysts of three genotypes (T3, T4 and T5) of *Acanthamoeba* to three commercially available drugs. The results showed that gentamicin and gatifloxacin showed statistically highly significant difference ($P < 0.001$) and levofloxacin showed statistically significant difference ($P < 0.05$) as compared with non-treated control. Of these three drugs, gentamicin was the most effective drug ($P < 0.001$). Gentamicin and gatifloxacin killed cysts of all three genotypes within 1

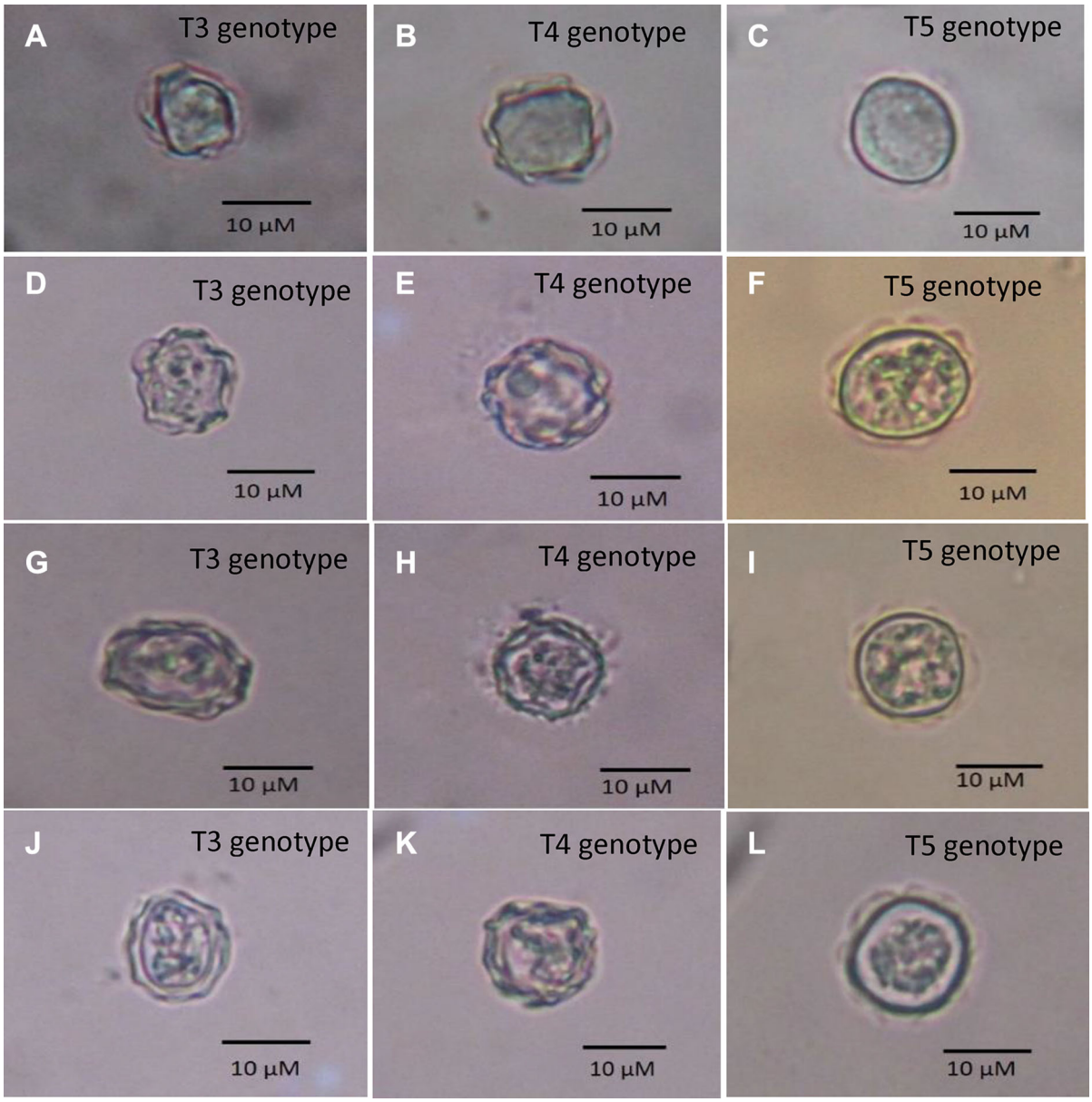

Fig. 3 Effect of each eyedrop against *Acanthamoeba* cysts of three genotypes after exposure for 24 h, then observed by light microscopy (× 40). **a–c** Untreated cyst, **d–f** treated with 0.02% chlorhexidine, **g–i** treated with 0.3% gentamicin, **j–l** treated with 0.3% gatifloxacin

and 24 h, respectively. In the case of levofloxacin, no genotypes were completely killed, even after 72 h of incubation. These drugs are primarily known for killing bacteria, but some of them have shown activity against *Acanthamoeba* spp. both in vitro and in clinical treatment [10, 16–18]. As used by us in vitro, drugs were diluted to 50% of their supplied concentration. In clinical use, drugs applied to the eye will also be diluted, and eventually flushed out, by the production of lachrymal fluid [19]. Gentamicin is a member of the aminoglycoside group, to have high efficacy against *Acanthamoeba* cyst by inhibiting protein synthesis [20]. We found that 0.3%gentamicin (3 mg/mL) has cysticidal activity after only 1 h. Another study has found that a lower concentration of gentamicin was also effective against clinical and

environmental strains of *Acanthamoeba* [16]. The mean minimum cysticidal concentration (MCC) at 30 °C in all tested strains was 0.193 mg/mL and 0.029 mg/mL at 37 °C (range 0.031–1 mg/mL) after incubation for 48 h. In addition, they found that environmental strains were more sensitive than clinical strains to gentamicin [16]. However, another study reported that gentamicin was clinically ineffective at 5 mg/mL but the drug's mean MCC was 13.33 mg/mL (range 10–20 mg/mL) after exposure to clinical strains for 24 h [10]. Gatifloxacin affected the morphology of cysts belonging to genotypes T3, T4 and T5 after 24 h. In this study, levofloxacin was the least effective of the three drugs tested against cysts, despite 72 h of incubation. Levofloxacin may require more incubation time to kill *Acanthamoeba* cyst. Levofloxacin is in the same group as gatifloxacin but produced different results. The possible explanation for this difference is the structure of gatifloxacin, fourth-generation fluoroquinolones, was modified by substitution of a methoxy group at position 8 of the quinolone ring, which allows for simultaneous inhibition of both DNA gyrase and topoisomerase IV. This modification was made to increase the efficacy against microorganisms more than the third-generation form, such as levofloxacin [21]. No previous study has reported the use of this agent alone in vitro. However, it was successful at 5 mg/mL as part of combined drug treatment of bacterial coinfection in an *Acanthamoeba* keratitis case [22].

We have presented data showing that commercially available ophthalmic drugs differ in their ability to kill *Acanthamoeba* cysts. Three different genotypes of *Acanthamoeba* were selected based on their potential to be pathogenic strains that can cause keratitis [1]. Although our results should be confirmed in vivo studies, gentamicin and gatifloxacin may be the optimum choices for treating the early stages of eye infection caused by *Acanthamoeba* spp. However, not only the efficacy of drugs should be considered in AK treatment, but also issues relating to the duration of treatment and the effects of dilution by the lachrymal fluid.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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